TWO PROTEINS FROM *USTILAGO MAYDIS*: PURIFICATION AND INTERACTION WITH DNA

BY

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1981

To my family.

ACKNOWLEDGMENTS

I would like to express my thanks to my advisory committee, Doctors Ken Berns, Bert Flanegan, Bill Hauswirth, Ed Siden, Dick Boyce and Nick Muzyczka. I am very grateful to my mentor Dr. Bill (ACE) Holloman for his friendship, excellent guidance and endurance throughout my graduate training. I would like to thank Dr. George Gifford for his academic and personal interest in my education.

An optimal environment for research was developed and maintained by Greg Chomic, Tom Rowe, Don Goodroe, Laura Prall, Michael Brougham, Sherin Smallwood, and Eric Kmiec. I am deeply indebted to these people for their patience, understanding and humor. I would especially like to thank Tom (TR, Tommy Wong, Johnny Ciringo) Rowe for his personal and academic companionship as well as his laugh. Thanks go to Sherin for her friendship, technical assistance and excellent graphic skills. Also I would like to thank Diane Blucher for her work on this manuscript.

The love and support of my family was an inspiration throughout this work. The constant encouragement of my parents John and Mary was indespensable. I also profited from the unique opportunity to have my grandparents Al, Marie, John and Mil for friendship and support.

Finally I would like to thank Dr. Marty Venneman for his advice to me in 1976, "Hell, why not go for it?"

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ABBREVIATIONS USED

ATP.....adenosine-5'-triphosphate

BSA.....bovine serum albumin

°C.....degree centigrade

Ci.....Curie (unit of radioactivity)

cpm.....counts per minute

d.....dalton

DBP.....DNA binding protein

DNA.....deoxyribonucleic acid

DNAse.....deoxyribonuclease

EDTA.....ethylenediamine tetra-acetic acid

form I.....negatively supercoiled, covalently closed DNA

form II.....circular duplex DNA containing a strand break

form III.....linear duplex DNA

form IV.....relaxed covalently closed DNA

g.....gram

h.....hour

HDP.....helix destabilizing protein

L.....liter

mamp.....milliampere

min.....minute

MOI.....multiplicity of infection

NAD.....nicotinamide adenine dinucleotide

PEG.....polyethylene glycol

poly [dA].....polydeoxyadenylic acid

poly [dG].....polydeoxyguanylic acid

poly [dT].....polydeoxythymidylic acid

psi.....pounds per square inch

RF I.....replicating form I

RNA.....ribonucleic acid

RNAse.....ribonuclease

rpm.....revolutions per minute

S.....Svedberg (unit of sedimentation)

SDS.....sodium dodecyl sulphate

SSC.....standard saline citrate
(0.15 M NaCl, 15 mM sodium citrate)

V.....volts

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

TWO PROTEINS FROM USTILAGO MAYDIS: PURIFICATION AND INTERACTION WITH DNA

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December, 1981

Chairman: William K. Holloman
Major Department: Immunology and Medical Microbiology

Cell extracts of Ustilago maydis were examined for proteins that alter the structure of DNA. Two proteins were isolated and characterized that interact with DNA. An activity that hydrolyzes DNA, termed nuclease β, was purified and the mode of DNA digestion by nuclease β was investigated. Also a DNA binding activity, termed DBP III, was isolated by utilizing the property of protein dependent retention of DNA on nitrocellulose filters. The in vitro characterization of these activities was performed to enrich the present understanding of eucaryotic DNA metabolism.

The single strand specific nuclease β has been purified 11,200-fold from crude extracts to about 70% homogeneity. The enzyme has a sedimentation coefficient of 4.3 S and a Stokes radius of 36Å consistent with a native form of a

single polypeptide of 68,000 daltons. The enzyme is fully active in the presence of chelating agents but is strongly inhibited by nucleotides. Maximal activity is seen at pH 6.0. The enzyme hydrolyzes linear DNA in an exonucleolytic fashion from the 5'-end liberating 3'-mononucleotides and small oligonucleotides. The enzyme is also active on both single stranded and duplex circular DNA, dephosphorylates 5'-mononucleotides, and hydrolyzes RNA.

A DNA binding activity, termed DBP III, was found to remain bound to DNA in the presence of 1.5 MNaCl. Purification of DBP III was accomplished by assaying for activity that retained DNA on nitrocellulose filters in the presence of 1.5 M NaCl. Purified preparations of DBP III contained one major polypeptide of 47,000 daltons. Protein binding to DNA required a minimum temperature of about 25°C. DBP III retained covalently closed DNA (either relaxed or negatively supercoiled) but did not retain nicked circular or linear DNA to nitrocellulose filters. Titration of form I DNA with ethidium bromide did not alter the amount of DNA bound by DBP III. DBP III-DNA complexes appeared to dissociate if the DNA was nicked after complex formation. Competition experiements suggest that DBP III has affinity for single stranded DNA, especially the homopolymer poly (dT). A plasmid containing an A·T rich DNA insert was also an effective competitor.

CHAPTER I
INTRODUCTION

The intimate association of DNA with protein confused early attempts at identifying which compound was the genetic material. The accumulation of experimental evidence from 1944 (Avery et al., 1944) to 1953 (Watson and Crick, 1953) indicated that deoxyribonucleic acid was the genetic material. Proteins possess the capacity to facilitate gene expression and replicate the genetic material. The molecular mechanisms by which proteins accomplish this manipulation of genetic material is the subject of extensive research. Although the secondary structure of DNA in vivo is probably a duplex right-handed helix, extensive tertiary structure is brought about by the interaction of proteins with DNA (Bak et al., 1977). It appears that the secondary and tertiary structure of DNA becomes altered during replication, recombination and repair. For example, replication of duplex DNA requires that the parent strands be unwound to allow them to act as templates (for example see Alberts and Sternglanz, 1977). Repair of DNA damage in the absence of light requires strand incision and removal of damaged bases (for review see Grossman et al., 1975; Hanawalt et al., 1979). Recombination between duplex molecules appears to occur through the breakage and reunion of DNA strands (Tomizawa, 1967; Meselson, 1967). A final example is that DNA replication in some eucaryotic viruses

seems to require nucleolytic incision of covalently linked DNA termini (Berns et al., 1978). Recent research on DNA metabolism has focused on the molecular mechanisms by which proteins make these alterations in DNA structure. One method used to identify the proteins involved in DNA metabolism is to isolate cell constituents and characterize their interaction with DNA in vitro.

A goal of this project was to isolate and characterize proteins that alter the structure of DNA. Previously isolated DNA binding proteins alter the structure of DNA by fundamentally different processes. The following discussion of proteins that bind to DNA provides a reference by which to compare a binding protein I have isolated from Ustilago maydis.

Proteins that Bind to DNA

Proteins that bind to DNA can most easily be separated into three types: i) proteins that bind to specific sequences in DNA ii) proteins that maintain the structure of chromatin and iii) proteins that alter the tertiary structure of DNA. Many of these proteins appear to be multifunctional and could be listed in more than one of the above categories.

Sequence Specific Binding Proteins

Proteins that bind to specific sequences in DNA have been isolated from both procaryotes and eucaryotes. These proteins seem to function in controlling gene expression and performing some functions in DNA replication. For example, the lac repressor of E. coli (Riggs et al., 1970) controls the

transcriptional activity of the lactose operon by binding to transcriptional operator sequences (for review see Beckwith and Zipser, 1970). Repressor protein-operator DNA complexes prevent the initiation of transcription of the lactose operon by RNA polymerase, another sequence specific binding protein (Hinkle and Chamberlin, 1972). The CI gene product of bacteriophage Lambda (Ptashne, 1971) and the lex A gene product of E. coli (Little et al., 1981; Brent and Ptashne, 1981) are also transcriptional repressor proteins that bind specific sequences. DNA restriction-modifiction enzymes (Modrich, 1979; Yuan, 1981) from procaryotic cells are another example of site specific binding proteins.

Eucaryotic cells also possess sequence specific binding proteins. For example, a binding protein isolated from Drosophila melanogaster (Hsieh and Brutlag, 1979a) binds specifically to sequences found in satellite DNA from this organism (Hsieh and Brutlag, 1979b). The eucaryotic virus SV-40 codes for a protein (gene A protein or T antigen) that binds to specific sequences on the viral genome (Reed et al., 1975). T antigen is a multifunctional protein thought to be involved in controlling both gene expression (Cowan et al., 1973) and replication (Tegtmeyer, 1972) of SV-40 DNA. Species specific DNA binding proteins have also been observed in eucaryotic cells that have been chemically transformed (Sen and Todaro, 1978).

Proteins that Maintain the Structure of DNA in Vivo

Proteins are thought to induce the compact structure of DNA *in vivo* (von Hipple and McGhee, 1972). The primary level of chromatin structure is maintained by a class of proteins called histones (Elgin and Weintraub, 1975). Histones are arranged on the DNA in approximately 200 base pair repeating units called nucleosomes (for review see Kornberg, 1977; McGhee and Felsenfeld, 1980). Examination of isolated chromatin at physiological ionic strength suggests that the nucleosomes further compact DNA by wrapping in a superhelix or solenoid (Bak *et al.*, 1977). The nonhistone chromosomal proteins also play a role in DNA compaction. Glycoproteins have been isolated from a variety of eucaryotic cells that may function in the condensation process of DNA *in vivo* (Champ *et al.*, 1978).

Chromosomal DNA that has been depleted of histones remains a compact structure (Ide *et al.*, 1975; Berezney and Coffey, 1976; Paulson and Laemmli, 1977). The DNA appears to be held in negatively supercoiled loops attached to a structure called the nuclear matrix (Benyajata and Worcel, 1976; Cook *et al.*, 1976; for review see Georgiev *et al.*, 1978). Proteins extracted from the nuclear matrix have been shown to bind DNA *in vitro* (Comings and Wallack, 1978). The experiments of Pardoll *et al.* (1980) suggest that newly replicated DNA is preferentially associated with the nuclear matrix. Thus the nuclear matrix may function in both maintaining the DNA superstructure of chromosomes and providing a site for DNA replication.

Proteins that Alter DNA Structure

The processes of DNA replication and recombination require that the secondary structure of DNA be altered. For example, the unwinding of duplex DNA is necessary for the semi-conservative replication of DNA (for review see Gefter, 1975; Alberts and Sternglanz, 1977; Champoux, 1978). Proteins termed helix destablizing proteins (HDP) have been isolated from a variety of procaryotic (Sigal et al., 1972; Alberts and Frey, 1970) and eucaryotic (Banks and Spanos, 1975; Herrick and Alberts, 1976; Schechter et al., 1980; Riva et al., 1980) cells. These binding proteins alter the temperature required for renaturation and denaturation of DNA in vitro (for example see Alberts and Frey, 1970).

Studies in vitro show that helix destabilizing proteins interact with other DNA enzymes. The HDP from T4 infected E. coli cells (T4 gene 32 protein) specifically stimulates the synthetic capacity of the T4 coded DNA polymerase (Huberman et al., 1971). Specific stimulation by HDP of the homologus polymerase has also been observed with protein from E. coli (Molineux et al., 1974), T7 infected cells (Reuben and Gefter, 1973), Ustilago maydis (Yarranton et al., 1976) and Hela cells (Riva et al., 1980). The formation of a physical complex between DNA polymerase and HDP has been demonstrated for proteins isolated from E. coli (Molineux and Gefter, 1975) and T4 (Huberman et al., 1971) or T7 (Scherzinger et al., 1973) infected cells. The HDP of E. coli also forms a complex with exonuclease I of E. coli.

Genetic studies with E. coli (Meyer et al., 1979; Johnson, 1977) and bacteriophage T4 (Breschkin and Mosig, 1977) suggest that helix destabilizing proteins are necessary for both replication and recombination in vivo. The Adenovirus HDP seems to be involved in both the initiation and chain elongation processes of DNA replication (van der Vliet et al., 1977).

Proteins that enzymatically alter the structure of DNA have also been isolated. The helical winding number of circular DNA can be changed by nicking and resealing the phosphate backbone of DNA with enzymes called topoisomerases (Wang and Liu, 1979). Nicking-sealing enzymes seem to be involved in transcription and some types of recombination (for review see Gellert, 1981).

Proteins that unwind DNA by utilizing the energy of ATP hydrolysis have also been isolated (Scott and Kornberg, 1978; Abdel-Monem et al., 1976; Mackay and Linn, 1976; Rosamond et al., 1979). The rep gene product of E. coli is an example of enzyme catalyzed unwinding of DNA chains (Duguet et al., 1978). Rep protein has been implicated in the process of chain elongation during replication of bacteriophage (Denhardt et al., 1972) and cellular (Lane and Denhardt, 1975) DNA.

Alterations in DNA Structure During Recombination

Homologous recombination is thought to occur by the exchange of strands between two duplex DNA molecules (Whitehouse, 1963; Holliday, 1964). The process of strand

exchange requires that the DNA duplexes be unwound and rewound to form a heteroduplex. The rec A protein of E. coli is a DNA-dependent ATPase that can reanneal homologous single strands of DNA (Weinstock et al., 1979; for review see Radding, 1981). Recent studies in vitro demonstrate that rec A protein can catalyze strand exchange between partially duplex molecules (Cunningham et al., 1980; Cassuto et al., 1980). The importance of enzyme catalyzed strand exchange is suggested by the observation that rec A deficient mutants have a severely reduced frequency of recombination (Clark, 1973). Rec A protein catalyzed strand exchange requires that one DNA molecule be at least partially single stranded and that either DNA molecule contain a free end (Cunningham et al., 1980; West et al.,1981). DNA damage induced recombination in the absence of replication appears to be initiated by the nucleolytic formation of nicks or gaps in the DNA (for review see Witkin, 1976; Howard-Flanders, 1973).

The preceding discussion illustrates that many types of structural alterations of DNA can be induced by protein-DNA interactions. Another source of protein induced alterations in DNA structure is the hydrolysis of the phosphate backbone of DNA by enzymes termed nucleases. Another goal of this project was to purify and characterize a nuclease activity I had observed in crude extracts of Ustilago maydis. The following is a discussion of the possible involvement of nuclease activities in DNA metabolism.

Nucleolytic Alterations in DNA Structure

The structural alteration of DNA by nucleolytic cleavage plays an important role in replication, repair and recombination. For example, the accumulation of progeny virus in bacteriophage øX174 infections requires the site specific cleavage of replicating form molecules by the øX174 gene A protein (Eisenberg et al., 1977). Another example is that nuclease activities associated with DNA polymerase I of E. coli appear to be important in replication and repair of DNA in vivo (for review see Kornberg, 1980). Nucleases that can hydolyze damaged DNA have been isolated from a variety of sources (Kushner and Grossman, 1971; Simon et al., 1975; Riazuddin and Grossman, 1977; for review see Grossman et al., 1975; Hanawalt et al., 1979). These damage specific nucleases probably stimulate recombination via the formation of nicked or gapped DNA molecules (see above).

Involvement of Nucleases in Recombination

Current models of recombination by strand exchange (Holliday, 1964; Meselson and Radding, 1975; Wagner and Radman, 1975) suggest a role for nucleases at multiple stages of the recombination process. The Meselson-Radding model (1975) proposes the incision of a duplex molecule that has assimilated a third DNA strand (D-loop). D-loop molecules have been formed nonenzymatically in vitro (Holloman et al., 1975). Specific cleavage of the displaced strand in D-loop molecules was demonstrated (Wiegand et al., 1977) using enzymes with single strand specific endonuclease activity

(i.e., SI nuclease or rec BC nuclease). The nicking of duplex DNA that has assimilated a third strand would allow for reciprocal strand exchange between the two molecules (Meselson and Radding, 1975). Experiments *in vivo* show that duplex circular DNA becomes nicked when cells also contain homologous DNA containing damage (Howard-Flanders *et al.*, 1978). The enzyme responsible for this "cutting in trans" activity has not been identified.

Strand exchange between DNA molecules that contain a single base difference could result in the formation of heteroduplex DNA containing a mismatched base-pair. The excision repair of mismatched bases is thought to result in gene conversion (Whitehouse, 1963; Holliday, 1964). Gene conversion describes the genetic observation of nonreciprocal allelic recombination. Single strand specific nucleases are candidates for this excision repair because of their ability to recognize structural distortions in duplex DNA. Cleavage of DNA containing structural distortions has been demonstrated with a variety of endonucleases including enzymes from Aspergillus (Wiegand et al., 1975), mung bean (Kowalski et al., 1976), T7 bacteriophage (Center and Richardson, 1970) and Ustilago (Holloman *et al.*, 1981). Ustilago mutants deficient in gene conversion were shown to have reduced levels of the single strand specific nuclease α (Holloman and Holliday, 1973).

Ustilago maydis as a Model System of Eucaryotic DNA Metabolism

Lower eucaryotes have proven to be valuable systems for the biochemical analysis of eucaryotic DNA metabolism. The ability to grow yeast and fungi on chemically defined media (solid or liquid) and the ability to isolate mutants in DNA metabolism have made these organisms popular systems for biochemical studies (for example see Holliday, 1974). The studies to be presented below were done with proteins isolated from the basidiomycete fungus Ustilago maydis.

A number of enzymes involved in DNA metabolism have been isolated from Ustilago maydis. For example, a DNA polymerase was isolated and characterized (Banks et al., 1976) from wild type cells and a mutant cell type that was temperature sensitive for DNA synthesis (Jeggo and Banks, 1975). The polymerase from the mutant strain was temperature sensitive for polymerizing activity suggesting this polymerase may be the major polymerase involved in chromosomal replication in U. maydis. A nuclease termed nuclease α has also been purified that appears to be involved in gene conversion (see above). Two proteins which bind to DNA have been isolated and characterized. DNA binding protein I (DBP I) was shown to be a helix destabilizing protein (Banks and Spanos, 1975). DBP I interacts with the DNA polymerase of Ustilago and reduces the Km of the polymerase for both substrate and template (Yarranton et al., 1976). DBP II from Ustilago can bind to DNA forming a protein-DNA complex that is soluble in dilute acid (Holloman, 1975;

Unrau et al., 1980). DBP II is a glycoprotein thought to be involved in the condensation process of nucleic acids in vivo (Champ et al., 1978).

Originally I was examining extracts of U. maydis for an activity that could catalyze strand assimilation of a DNA fragment into a homologous duplex DNA molecule. During these investigations I observed a nuclease activity and DNA binding activity. The work described in the subsequent chapters deals with a nuclease, termed nuclease β, and a DNA binding protein termed DBP III. The goals of this project were to purify the nuclease and DNA binding protein activities and characterize their interaction with DNA. Some of this work has previously been published (Rusche et al., 1980).

CHAPTER II
METHODOLOGY

Cell Culture

Strains

Ustilago maydis a_1, b_2 was obtained from Dr. Robin Holliday, National Institute for Medical Research, London. Cells were grown in a medium composed of 2% peptone, 2% sucrose, and 1% yeast extract in a Vir-Tis fermentor under vigorous aeration. When the density reached 5 X 10^7 cells/ml the cells were harvested by centrifugation and stored frozen at -70°C. Bacteriophage øX174 am3 and its host *Escherichia coli* HF4704 were obtained from Dr. Charles Radding, Yale University. Phage P22 and its host *Salmonella typhimurium* DB25 were obtained from Dr. David Botstein, Massachusetts Institute of Technology. *Escherichia coli* HB101 containing the plasmid aDM23-24 (Hsieh and Brutlag, 1979a) was obtained from Dr. Douglas Brutlag, Stanford. HB101 containing pBR322 (Bolivar *et al.*, 1977) was obtained from Dr. Susan Lederberg, Stanford University.

Media

Minimal media was used for the *in vivo* labeling of phage DNA. The P22 phage host cell strain DB25 was grown in LCG medium (Botstein, 1968). This medium contained 0.5 g NaCl, 8 g KCl, 1.1 g NH_4Cl, 5g casein hydrolysate, 0.12 g $MgSO_4$, 0.055 g $CaCl_2$, 12.1 g Tris base, and 10 µg of $FeSO_4$ per liter of

solution and was adjusted to pH 7.5. The ØX174 phage host cell strain HF4704 was grown in TPA medium (Benbow et al., 1974). This medium contained 12.1 g Tris base, 2 g casein hydrolysate, 1 g NH_4Cl, 0.3 g $MgSO_4$, 0.24 g NaCl, 0.3 g KCl, 0.22 g $CaCl_2$, and 10 µg of $FeSO_4$ per liter of solution and was adjusted to pH 7.5. These media were supplimented with glucose, thymine and phosphate as indicated below. Strains of E. coli HB101 containing plasmid DNA were grown in Luria broth medium that contained 5 g NaCl, 10 g tryptone, and 5 g yeast extract per liter. The pH of the media was adjusted to 7.5 with 4.5 mmol of NaOH.

Nucleic Acid Preparation

Isolation of DNA Made in Vivo

Bacteriophage P22 DNA was made as described by Carter and Radding (1971). Strain DB25 was grown in 1 liter of LCG medium supplimented with 1 mM KPO_4, 0.2% glucose, and 3 mg/ml thymine. When the cell density reached 4 X 10^8 cells/ml the culture was infected with P22 phage at a multiplicity of infection (MOI) of 5. Five milliCurie of [^{3}H] thymidine was added and incubation continued for 2.5 h. DNAse and RNAse (100 µg of each) were added and the solution incubated an additional 10 min. To this mixture was added 29 g of NaCl and 110 g of PEG 6000 and the mixture was stored at 4°C overnight. The resulting precipitate was collected. This was washed and pelleted three times with 10 mM Tris-HCl, pH 7.5, collecting the supernatant each time. The phage was then banded in a CsCl step gradient of 1.35 g/ml and 1.5 g/ml. The DNA was obtained by phenol extraction followed by ethanol precipitation.

Bacteriophage øX174 DNA was made using a method described by Beattie et al. (1977). Strain HF4704 was grown in TPA medium supplimented with 3 µg/ml thymine, o.2% glucose, and 1 mM KPO_4. At a cell density of 5 X 10^8 cells/ml øX174 am3 phage was added at a MOI of 10. At 3 min. post infection 60 mg per liter of chloramphenicol was added and after an additional 7 min., 5 mCi of [^{3}H] thymidine. This was incubated for 2 h. and the cells were pelleted. The cells were resuspended in 15 ml of a solution containing 50 mM Tris-HCl 7.5, 10 mM EDTA, and 10% sucrose. To this solution was added 30 mg of lysozyme and the cells were incubated on ice for 15 min. Then 0.8 ml of a 10% sarkosyl solution was added and the mixture was centrifuged for 1 h. at 25,000 rpm in a SW27 rotor. The supernatant was phenol extracted and ethanol precipitated. The precipitate was resuspended in 2 ml of 10 mM Tris, 1 mM EDTA and treated with 0.1 mg of RNAse for 15 min. The RFI DNA was isolated by centrifugation through a 35 ml 5-20% sucrose gradient for 18 h. at 25,000 rpm in a SW27 rotor. One milliliter fractions containing radioactivity were pooled and precipitated with 2 volumes of ethanol.

HB101 cells containing a plasmid were grown in 1 liter of Luria broth medium to a density of 4 X 10^8 cells/ml. Chloramphenicol was added to 150 µg/ml and the culture incubated 18 h. (Clewell, 1972). The cells were pelleted and the plasmid DNA purified as described for øX174 RFI DNA. As an alternative to the final step of sucrose gradient sedimentation, the form I DNA was purified by chromatography through

an affinity column containing acridine yellow (Boehringer Mannheim). After RNAse treatment the solution was diluted to 10 ml with a buffer containing 0.5 M NaCl and 20 mM Na citrate, pH 6.0 as suggested by D. Rawlins of this department. This was applied to an acridine yellow column (20 ml bed volume) equilibrated with 0.5 M NaCl, 20 mM Na citrate, pH 6.0 and washed with the same buffer (100 ml). The RFI was eluted by washing the column with 20 mM Na citrate, pH 6.0, containing 1.5 NaCl. This wash was collected in 5 ml fractions and DNA eluted in fractions 5 and 6. This DNA was dialyzed versus 10 mM Tris-Hcl 7.5, 1 mM EDTA and precipitated with ethanol.

Modification of DNA Substrates

The units indicated for enzymes obtained commerically are those described by the manufacturer. Linear DNA (RFIII) was prepared by cleavage of øX174 RFI DNA with restriction endonuclease Pst I. One µmole of øX RFI DNA was incubated with 25 units of Pst I at 30°C for 8 h. in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol. The 5'-^{32}P-labeled DNA was prepared by dephosphorylating DNA with bacterial alkaline phosphatase and rephosphorylating with polynucleotide kinase in the presence of [γ-^{32}P] ATP as described by Weiss et al. (1968). A reaction mixture (0.5 ml) containing 0.5 µmol of dephosphorylated DNA, 1 mM [γ^{32}P] ATP (200 mCi/nmol), 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 units of polynucleotide kinase was incubated at 37°C. After 1 h. EDTA was added to 20 mM and the mixture was held at 65°C for 15 min. This was applied to a Sephacryl S-200 column (10 ml

bed volume) equilibrated with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl and the unincorporated [γ-^{32}P] removed from the labeled polynucleotide. DNA was precipitated with ethanol and resuspended in 10 mM Tris, pH 7.5, and 1 mM EDTA. The 3'-^{32}P-labeled DNA was prepared as described by Nossal and Hershfield (1971). A reaction mixture (0.3 ml) containing 0.2 µmol of øX174 RFIII DNA, 100 µM [α-^{32}P] dGTP (10 µCi/nmol), and 5 units of T4 DNA polymerase in 67 mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 15 mM $(NH_4)_2SO_4$ was incubated at 15°C for 30 min. The reaction was terminated by addition of EDTA to 10 mM and heating at 65°C for 15 min. The DNA was purified by chromatography through Sephacryl S-200 as described above. When necessary, DNA was denatured by heating to 100°C for 2 min.

^{32}P-labeled deoxynucleotides were prepared by exhaustive digestion of [^{32}P] DNA with panceatic DNAse and snake venom phosphodiesterase. A reaction mixture containing 0.46 µmol of øX[^{32}P] DNA (2 X 10^4 cpm/nmol) and 25 µg of pancreatic DNAse in 10 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ was incubated at 37°C. After 30 min. the mixture was brought to pH 9.0 by addition of sodium glycinate buffer and 10 units of snake venom phosphodiesterase were added. The reaction was terminated after 60 min. by the addition of a stoichiometric amount of EDTA followed by heating at 100°C for 10 min. All nucleic acid concentrations are expressed as nucleotide residues.

Relaxed convalently closed øX174 DNA (RF IV) was prepared using partially purified Ustilago topoisomerase (Rowe et al.,

1981). A reaction mixture (0.25 ml) containing 200 nmol of [^{3}H] øX174 RFI DNA, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 300 units topoisomerase was incubated at 22°C for 1 h. One unit of topoisomerase is that amount necessary to relax 1 nmole of RFI DNA in 1 h. EDTA was then added to 20 mM and the solution extracted with phenol and the DNA precipitated with ethanol.

Assays

Nuclease Assays

The deoxyribonuclease assay measures the conversion of DNA to an acid soluble form. Standard assay mixtures (0.1 ml) containing 0.1 M potassium acetate, pH 6.0, and about 5 nmol of denatured P22 [^{3}H] DNA (1-3.5 X 10^4 cpm/nmol) were incubated with enzyme at 37°C for 30 min. Reactions were terminated by addition of 0.4 ml ice cold carrier salmon sperm DNA (1 mg/ml) and 0.5 ml of 5% trichloroacetic acid. Tubes were held on ice for 10 min., then centrifuged at 8000 rpm for 10 min. An aliquot (0.5 ml) of supernatant was removed and counted in 5 ml Triton scintillation fluor (New England Nuclear, Formula 950A). One unit of activity is that amount of enzyme necessary to render 1 nmol of DNA acid soluble under these conditions. The activity was proportional to enzyme concentration at levels of 0.01 to 0.25 units. The extract was diluted when necessary with buffer containing 50 mM Tris-HCl pH 7.5, 0.1 mg/ml bovine serum albumin, 50% glycerol.

The endonuclease assay measures the conversion of superhelical ØXRFI[^{3}H] DNA (1-4 X 10^4 cpm/nmol) to an open "nicked" form and was performed as described by Kuhnlein et al. (1976). One unit is defined as the amount of enzyme required to cause retention of 1 nmol of DNA (as nucleotide) on a nitrocellulose filter.

A nuclease β digest of denatured [^{3}H] DNA was redigested with snake venom phosphodiesterase with or without prior treatment with alkaline phosphatase. If the oligonucleotides contained 3'phosphates the phosphatase treatment would make them a more suitable substrate for the venom phosphodiesterase (Turner and Khorana, 1959). In an experiment performed by William Holloman, a reaction mixture (0.12 ml) containing 63 nmol ØX174 [^{3}H] DNA (2.7 X 10^4 cpm/nmol) in 0.1 M sodium acetate buffer, pH 6.0, was incubated with 20 units of nuclease β. After 30 min. at 37°C, 0.1 ml of 10% trichloroacetic acid was added and the mixture was held on ice for 10 min. After centrifugation, the supernatant was discarded and the pellet was washed with 1 ml of 0.02 M HCl. The precipitated DNA was dissolved in 50 1 of 0.1 M NaOH and the solution neutralized by addition of 5 µ l of 0.1 M Tris-HCl, pH 8.0 and 5µl of 1.0 M HCl. The mixture was split into two equal portions each containing 18 nmol of digested DNA. One portion was dephosphorylated by incubation at 65°C for 10 min. with 2 µg of bacterial alkaline phosphatase. The activities of the treated and untreated DNA samples as substrates for snake venom phosphodiesterase were then determined. Reaction

mixtures (1.0 ml each) containing 50 mM Tris-HCl, pH 8.8, 10 mM $MgCl_2$, 10 mM NaCl, 1.7 µg snake venom phosphodiesterase, and either the dephosophorylated or untreated DNA samples were incubated at 37°C. Initial rates of digestion were determined by removing aliquots of 0.2 ml at intervals and measuring the amount of DNA made acid soluble. Reactions were linear for 10 min.

5'-Nucleotidase Assay

5'-Nucleotidase activity was assayed by measuring the conversion of ^{32}P-labeled deoxynucleotides to a form no longer able to adsorb to norite. Reaction mixtures were the same as described for the deoxyribonuclease assay except that 5 nmol of ^{32}P-labeled 5'deoxynucleotides (0.5-2 X 10^4 cpm/nmol) were used in lieu of DNA. Reactions were terminated by addition of 0.3 ml bovine serum albumin (2 mg/ml), 0.3 ml salmon sperm DNA (1 mg/ml), and 0.3 ml of norite (20% w/w) in 1 M HCl, 50 mM KH_2PO_4. Norite was removed by centrifugation and an aliquot (0.5 ml) of the supernatant was removed and counted in Triton fluor.

Protein Binding to DNA

The binding of protein to DNA was observed by measuring the retention of labeled DNA on a nitrocellulose filter (Riggs et al., 1970). A reaction mixture (0.1 ml) containing 0.1 M potassium acetate, 1 mM EDTA, 1 nmol [^3H] DNA (2 X 10^4 cpm/nmol), and extract was incubated at 37°C for 10 min. The reaction was terminated with 1 ml of 5 X SSC (Standard Saline Citrate: 0.15 M NaCl, 15 mM Na citrate)

and the solution was filtered through BA85 nitrocellulose filters at a flow rate of 3 ml/min. The filters were rinsed with two 1 ml aliquots of 5X SSC and dried. Radioactivity remaining on the filters was counted in 2 ml of a toluene fluor (New England Nuclear Econoflouor). For labeled single stranded DNA the filters were treated with 0.5 M KOH for 15 min. prior to use. The filtration buffer used was 0.5 X SSC.

The DNA from the above reaction could be directly visualized by gel electrophoresis. Identical 0.1 ml reaction mixtures were loaded directly to a 1.4% agarose gel containing 5 mM sodium acetate, 40 mM Tris-HCl, pH 7.9 and 1 mM EDTA. Gels were run at 40V and 25 mamp overnight and DNA visualized by staining with ethidium bromide.

Protein Determinations

The amount of protein was determined using the method of Bradford (1976) using bovine serum albumin as a standard. Low amounts of protein (< 0.025 mg/ml) were determined by measuring the absorbance of 280 nm light in a Zeiss PMQ III spectrophotometer. Protein was calculated using $\varepsilon_{280}^{0.1\%} = 1.0$.

Gel Electrophoresis of Proteins

Electrophoresis of native enzyme was carried out with the discontinuous buffer system described by Reisfeld *et al.* (1962) in tube gels. Running gels (3 X 70 mm) containing 5.0% acrylamide, 0.2 N,N'-methylenebisacrylamide were overlaid with a 10 mm stacking gel. A sample of 100 µl containing 10 µg cytochrome *c* as marker was added and a current of

3 mamp was applied until the cytrochrome c had migrated to the bottom of the gel. Cooling was provided by circulating ice water around the gel during the course of the run.

Isoelectric focusing of protein samples was performed in polyacrylamide tube gels (O'Farrell, 1975). The gels (3 X 70 mm) contained 5% acrylamide, 0.15% N,N'-methylenebisacrylamide, 2% ampholines (Pharmalyte, pH 3-10), and 0.03% ammonium persulfate. Protein samples (100 µl) made 1% in ampholines were layered on top of the gel. The cathode buffer (60 mM sulfuric acid) and the anode buffer (40 mM NaOH) were added to the reservoirs and the samples electrophoresed for 15 min. at 75 volts. Electrophoresis was continued for 30 min. at 350 volts. Two parallel gels were electrophoresed and both were cut into 2.5 mm slices. The slices from one gel were soaked in 0.5 ml of H_2O and the pH of the eluates determined. The slices from the other gel were eluted in 100 µl of 50 mM Tris-HCl, pH 7.0, 0.1 mg/ml BSA, 1 mM EDTA and 10% glycerol overnight at 4°C. These eluates were assayed for single stranded DNA hydrolysis activity.

Electrophoresis in the presence of sodium dodecyl sulfate was done according to Laemmli (1970) in slab gels of 10% acrylamide, 0.27% N,N'-methylenebisacrylamide. Gels were fixed in a solution of 40% ethanol, 7% acetic acid, stained with Coomassie brilliant blue and destained by soaking overnight in several changes of 5% ethanol, 7% acetic acid.

Protein Purification

Purification of Nuclease β

Wild type haploid cells of Ustilago maydis a_1 b_2 were grown as described earlier. All operations were carried out at 0°-4°C. Frozen cells (100 g) were suspended and thawed in 200 ml of 50 mM Tris-HCl, pH 7.5, 10% glycerol and sonicated for 1 minute in order to disperse clumped cells. The cells were crushed at 20,000 psi by passage through a French pressure cell and the broken cell suspension was centrifuged at 14,000 rpm for 30 min. in a JA 20 rotor of a Beckman J21-C centrifuge (Fraction I). Floating lipid was removed from the supernatant and 68 g of solid ammonium sulfate were added. After 30 min. the solution was centrifuged at 10,000 rpm for 10 min. Additional ammonium sulfate (30 g) was added and after 90 min. the precipitate that formed was removed by centrifugation. The supernatant (Fraction II) was applied directly to an Octyl-Sepharose column (50 ml bed volume) previously equilibrated with a 70% saturated ammonium sulfate solution containing 50 mM Tris-HCl, pH 7.5, 10% gylcerol. After the column was washed with 100 ml of equilibration buffer, the enzyme was eluted with the same buffer but 30% saturated with ammonium sulfate. Fractions of 7 ml were collected and those containing activity greater than 50 units/ml were pooled and dialyzed against three 2 L changes of 20 mM potassium phosphate pH 7.5, 10% glycerol (Fraction III). A column of Affi-Gel Blue agarose (20 ml bed volume) equilibrated with the phosphate buffer was loaded with Fraction III,

washed with 100 ml of buffer, followed by a linear gradient from 0 to 0.7 M NaCl (200 ml total). A single peak of activity eluted halfway through the gradient. Fractions containing activity greater than 50 units/ml were pooled and dialyzed against 2 L of 20 mM potassium phosphate pH 7.5, 10% glycerol (Fraction IV) and loaded onto a column of phosphocellulose (7 ml bed volume) equilibrated with the same buffer. The column was washed with 50 ml of phosphate buffer followed by 100 ml of a linear gradient from 0 to 0.6 M NaCl. The enzyme eluted as a single peak of activity at 0.3 M NaCl (Fraction V). Fraction V was applied directly to a column of hydroxylapatite (1.5 ml bed volume) equilibrated with 20 mM potassium phosphate pH 7.5, 10% glycerol. The column was washed with 20 ml of buffer and then 40 ml of a linear gradient of potassium phosphate buffer pH 7.5 (0.02 to 0.75 M) containing 10% glycerol. Fractions (1.5 ml) were collected into polyallomer tubes to minimize loss due to adsorption to glass. Activity emerged as a single peak at 0.3 M potassium phosphate. The peak fractions were pooled and dialyzed against 20 mM Tris-HCl pH 7.5, 50% glycerol (Fraction VI).

Purification of DNA Binding Protein III

Ustilago maydis cell (100g) strain a_1 b_2 were suspended in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA) that contained 1 M NaCl. Cells were broken by passage through a French pressure cell at 20,000 psi. Cell debris was removed by centrifugation and the resulting supernatant of 210 ml (Fraction I) was made 9% in polyethylene glycol (PEG 6000). After 15 min. on ice the precipitate that formed was removed

and the supernatant dialyzed versus three 2 L changes of buffer A. The dialyzed solution of 400 ml (Fraction II) was applied to a 200 ml column of DEAE-cellulose equilibrated with buffer A. Two column volumes of buffer A were washed through the column followed by a linear 2 L gradient of 0-0.5 M NaCl in buffer A. DNA binding activity eluted from 50 to 150 mM NaCl and these fractions were pooled. The pooled activity was dialyzed versus 2 L of buffer A yielding a volume of 335 ml (Fraction III). A 32 ml phosphocellulose column equilibrated in buffer A was used to further purify the DNA binding activity. After passing the extract through the phosphocellulose the column was washed with 100 ml of buffer A and eluted with a 300 ml 0-0.4 M NaCl gradient containing buffer A. A single peak of activity emerged between 100 and 150 mM NaCl. Fractions containing activity were pooled yielding a volume of 70 ml (Fraction IV). Half of the phosphocellulose pool was concentrated ten-fold by bringing the solution to 60% saturation in ammonium sulfate and collecting the resulting precipitate. This pellet was resuspended in 3.4 ml of buffer A containing 20% glycerol and applied to a Sephacryl S-300 column. The column of 290 ml had an exclusion volume of 117 ml as determined using blue dextran. Cytochrome c eluted after 270 ml were collected. Three milliliter fractions were collected and the activity found to elute in the excluded volume. The three peak fractions were pooled and stored (Fraction V). Binding activity in this fraction was stable for at least one month when stored at 4°C.

Materials

Chromatographic Media and Chemicals

Affi-Gel Blue, hydroxylapatite agarose and Dowex-1 (X8) were purchased from Bio-Rad Laboratories. Octyl-Sepharose, Pharmalytes (pH 3-10), Sephacryl S-200 and S-300 were from Pharmacia Fine Chemicals; DEAE-cellulose and phosphocellulose were from Whatman. Ethidium Bromide, activated charcoal and sodium dodecyl sulfate (SDS) were from Sigma. Sarkosyl NL 30 was from ICN Parmaceuticals. Scintillation fluors Econofluor and Formula 950-A were from New England Nuclear. Nictrocellulose filters type BA85 were purchased from Schleicher & Schuell.

Enzymes and Radioisotopes

Pancreatic DNAse, bacterial alkaline phosphatase and snake venom phospodiesterase were purchased from Worthington Biochemicals Inc. Nuclease P1 from Penicillium citrinum was purchased from Calbiochem-Behring Corp; Snake venom 5'-nucleotidase, bovine serum albumin, lysozyme, cytochrome c and the synthetic polynucleotides of dT and dG were from Sigma Chemical Co. Restriction endonuclease Pst 1 and polynucleotide kinase were from Bethesda Research Laboratories. Poly [dA] was obtained from Miles. T4 DNA polymerase was prepared as described by (Nossal and Herschfield, 1971). Ustilago topoisomerase (hydroxylapatite fraction; Rowe et al., 1981) was gift a from Tom Rowe and Michael Brougham; [^{3}H] thymidine was from Schwarz Mann; [^{32}P] orthophosphate, [γ-^{32}P] ATP and [α-^{32}P] dGTP were from New England Nuclear.

CHAPTER III
PURIFICATION AND CHARACTERIZATION OF NUCLEASE β

Nucleases have been implicated in recombinational processes in both procaryotic and eucaryotic cells (see Chapter I). In vitro characterization of nucleases can often explain observations of normal or mutant strains. For example, a recBC mutant of E. coli (recB270, recC271) demonstrates a reduced recombination frequency at 43°C. Purification of the recBC coded nuclease from this mutant revealed that only the ATP-dependent exonuclease activity was thermolabile (Kushner, 1974). Protein purification and characterization made possible the correlation of a specific phenotype with a defined enzymatic activity.

This project was aimed at isolating and characterizing a nuclease from Ustilago maydis. A nuclease active on single stranded DNA has previously been isolated and characterized from U. maydis (Holloman and Holliday, 1973). The nuclease, termed nuclease α, shows increased activity upon duplex DNA substrates containing structural distortions (Holloman et al., 1981). Ustilago mutants deficient in spontaneous or damage induced allelic recombination (gene conversion) were found to have reduced amounts of nuclease activity (Badman, 1972). In particular, these mutant cells had reduced levels of nulease α (Holloman and Holliday, 1973).

Nuclease activity was examined in extracts of U. maydis that had been fractionated by chromatography on DNA-cellulose. A single strand specific nuclease activity was observed that had different characteristics than the previously isolated nuclease α. This chapter describes the purification and characterization of this enzyme, termed nuclease β.

Purification

Fractionation of Nuclease β

The assay used to purify nuclease activity measures the conversion of [^{3}H] DNA to a form soluble in dilute acid (see Chapter II). Nuclease β was purified by the fractionation procedures outlined in table I. A detailed description of enzyme isolation is given in Chapter II. Most proteins (90%) present in crushed cell extract could be precipitated by bringing the solution to 70% saturation in ammonium sulphate. Nuclease activity remaining in the supernatant chromatographed as a single peak of activity on all the subsequent steps in purification. Ammonium sulphate fractionation of crude extracts would allow rapid screening of mutant strains for nuclease β. The highly concentrated salt solution containing nuclease activity was applied directly to an Octyl-Sepharose column. Nuclease was eluted by washing the column with buffer containing 30% ammonium sulphate. The eluate was dialyzed to remove the salt and applied to an Affigel-Blue agarose column. Enzyme activity was eluted with a NaCl gradient and the fractions containing nuclease were pooled and dialyzed. Nuclease β was furhter purified by chromatography on phosphocellulose

TABLE I. Purification of Nuclease β

	Fraction	Volume	Activity	Protein	Specific Activity	Purification	Yield
		ml	units	mg	units/mg		%
I.	Crude	235	57,600	4,510	12.8	1	100
II.	Ammonium sulfate	260	62,300	546	114	9	108
III.	Octyl-Sepharose	58	48,100	348	138	11	83
IV.	Affi-Gel blue	63	22,600	23.3	970	81	39
V.	Phosphocellulose	21	17,700	0.64	25,600	2,000	31
VI.	Phydroxylapatite	3.4	17,000	0.12	143,000	11,200	30

100 g of U. maydis cells were used as starting material. A detailed description of the procedure is given in Chapter II. All subsequent experiments were performed with Fraction VI enzyme.

and hydroxylapatite. This fractionation procedure purified nuclease β to a specific activity of 142,000 units/mg protein. The enzyme has been stable for 2 years at -20°C.

Homogeneity

Fraction VI represents an 11,200-fold increase in specific activity compared to crude extracts. Electrophoresis of purified nuclease in acrylamide gels under nondenaturing conditions revealed two major protein bands after the gel was stained with Coomassie blue (see Chapter II). The slower migrating band, which accounted for approximately two-thirds of the stain, comigrated with nuclease activity (figure 1). Further chromatography of fraction VI upon DNA-cellulose, carboxymethyl cellulose or QAE-Sephadex did not separate the two proteins present in this fraction. Storage of fraction VI for 6 months caused a change in the relative amounts of the two bands visible after native gel electrophoresis. The faster migrating band now contained about 60% of the Coomassie stain. Perhaps the contaminant observed by native gel electrophoresis arises from proteolytic degradation of the band containing nuclease activity.

Molecular Size and Isoelectric Point

The band from native gel electrophoresis that co-migrated with nuclease activity was electrophoresed under reducing conditions in a SDS-gel (see Chapter II). The protein migrated with a molecular weight of 68,000 when compared with standard proteins electrophoresed in parallel (figure 2). Purified nuclease β had a sedimentation coefficient of 4.3 S (figure 3)

Figure 1

Gel electrophoresis of nuclease β. Parallel tube gels loaded with equal aliquots of Fraction VI were prepared and electrophoresed for 3 hrs as described in Chapter II. After the run was complete one gel was cut into 2 mm slices and each slice eluted for 18 hrs at 4° in a small volumne (100μl) of 50 mM Tris-HCl pH 7.5, 10% glycerol. Deoxyribonuclease activity (o), endonuclease activity (●) and 5'-nucleotidase activity (Δ) were then determined. A parallel gel was stained for protein. Mobilities indicated are relative to cytochrome c, the heavily stained band with R_f of 1.0.

Activity (units/slice)
Nucleotides or Pi Liberated
30
15
0.2
0.4
0.6
0.8
1.0
Rf
2.0
1.0
Endonuclease x 10-2
Activity (units/slice)

Figure 2

SDS-gel electrophoresis of nuclease β. Fraction VI was subjected to discontinuous gel electrophoresis and the gel was sliced as described in the legend to fig. 1. The slices corresponding to those containing nuclease β activity as determined in a parallel gel were eluted in 50 mM Tris-HCl pH 6.8, 5% 2-mercapto-ethanol, 2% SDS, 10% glycerol, the eluates pooled and boiled for 2 min.and subjected to electrophoresis as described in Chapter II. Parallel slots contained the following protein standards: phosphorylase b, bovine serum albumin, ovalbumin, chymotrypsinogen, soybean trypsin inhibitor. The arrow indicates where the nuclease β protein band migrated. Mobilities are relative to a bromphenol blue tracking dye.

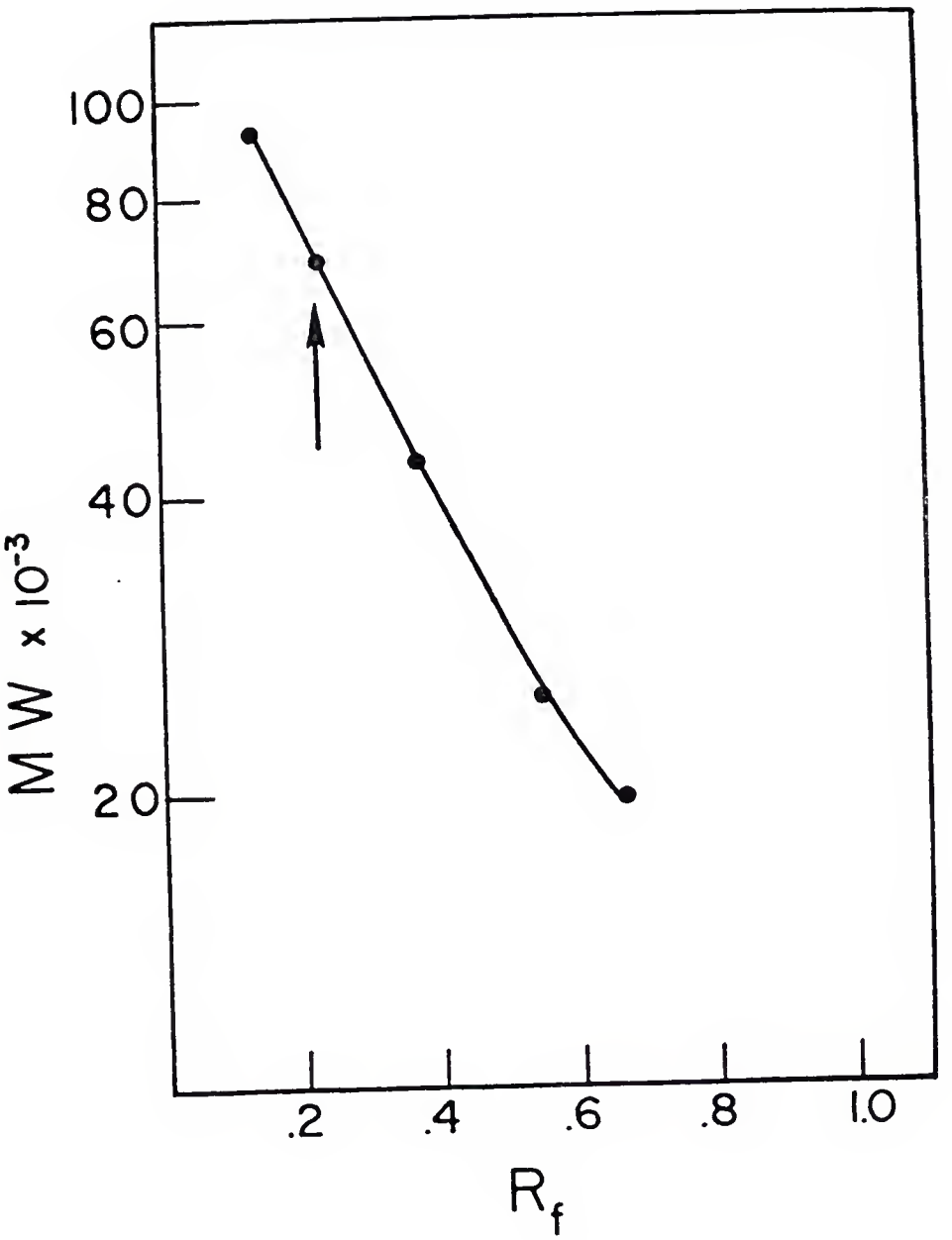
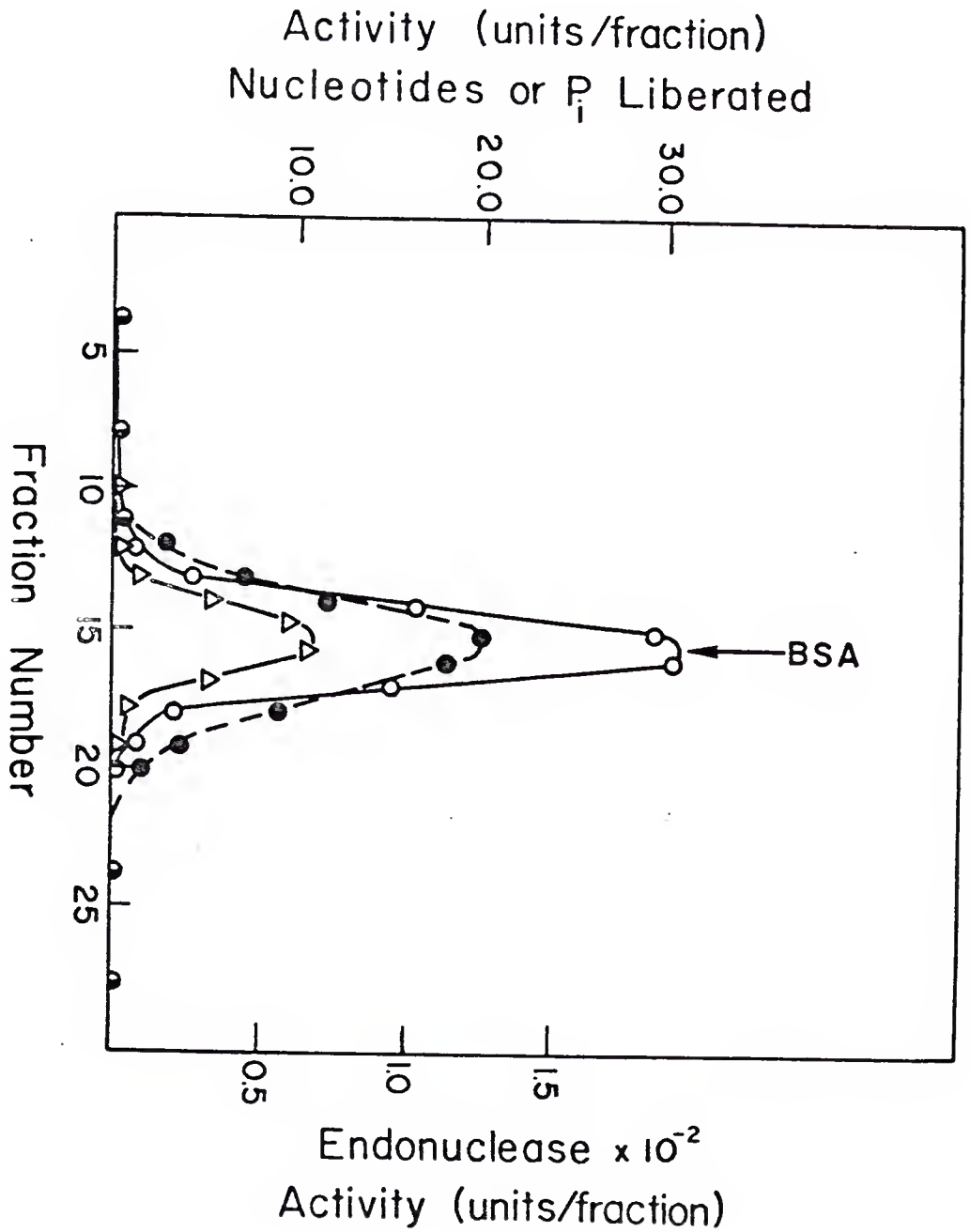

100
80
60
40
20
MW x 10⁻³
.2
.4
.6
.8
1.0
Rf

Figure 3

Centrifugation of nuclease β in a glycerol gradient. A portion of the purified enzyme (200 units) together with 1 mg of bovine serum albumin in 0.2 ml 50 mM Tris-HCl pH 7.5 were layered on top of a 5 ml 20 to 40% gradient of glycerol containing 50 mM Tris-HCl pH 7.5, 0.1 M NaCl. Centrifugation was for 35 hr at 45,000 rpm and 4°C in the Beckman SW50.1 rotor. At the end of the run fractions were collected from a hole punched in the bottom of the tube and each fraction assayed for deoxyribonuclease (o), endonuclease (●), and 5'-nucleotidase (Δ) activities. Sedimentation is from right to left.


Activity (units/fraction)
Nucleotides or P_i Liberated
10.0
20.0
30.0
Fraction Number
5
10
15
20
25
BSA
0.5
1.0
1.5
Endonuclease x 10^{-2}
Activity (units/fraction)

as determined by centrifugation through glycerol gradients (Martin and Ames, 1961). The Stokes radius of the native enzyme was determined by gel filtration (Siegal and Monty, 1966) to be 36Å (figure 4). An examination of the size, shape and electrophoretic properties of nuclease β suggests the enzyme exists in solution as a globular polypeptide of 68,000 daltons. An isoelectric point of pH 5.7 (figure 5) was determined by electrophoresis of nuclease β in acrylamide gels containing ampholines (O'Farrell, 1975).

Reaction Conditions

Optimum Conditions for Hydrolysis of DNA

Nuclease β was highly active on single stranded DNA. Denatured DNA was hydrolyzed about 1,000 times faster than native duplex DNA (figure 6). The optimum pH for nuclease activity in acetate, imidazole, or 2(N-morpholino) ethane sulfonate buffers was 6.0 (figure 7). Nuclease β was most active in sodium or potassium acetate. Enzyme activity in acetate buffer at pH 5.0 or sodium piperazine-N,N'-bis[2 ethane sulfonic acid] buffer at pH 7.0 was only half the amount observed at pH 6.0. The enzyme was not active in Tris-HCl buffer at pH 7.0.

Nuclease β was active over a wide range of temperature (figure 8A) with maximal activity at 42°C. DNA hydrolysis by the enzyme was optimal at an ionic strength of 0.2 M potassium acetate (figure 8B). The concentration of the monovalent cation appeared to be the critical component. The amount of nuclease activity in 0.2 M potassium acetate was identical to that observed in 0.05 M potassium acetate that

Figure 4

Determination of Stokes radius for nuclease β. A column of Sephacryl S-200 (2.5 X 60 cm) was equilibrated with 50 mM potassium phosphate pH 7.5, 0.2 M KCl, 10% glycerol. The excluded volume of 124 ml and the included volume of 287 ml were determined with the use of ^{3}H-labeled øX174 phage and $^{32}PO_4^{-3}$. Proteins used to calibrate the column included catalase, bovine serum albumin, ovalbumin, pancreatic deoxyribonclease, and cytochrome c. Approximately 200 units of nuclease β were loaded on the column. Recovery was 5%.

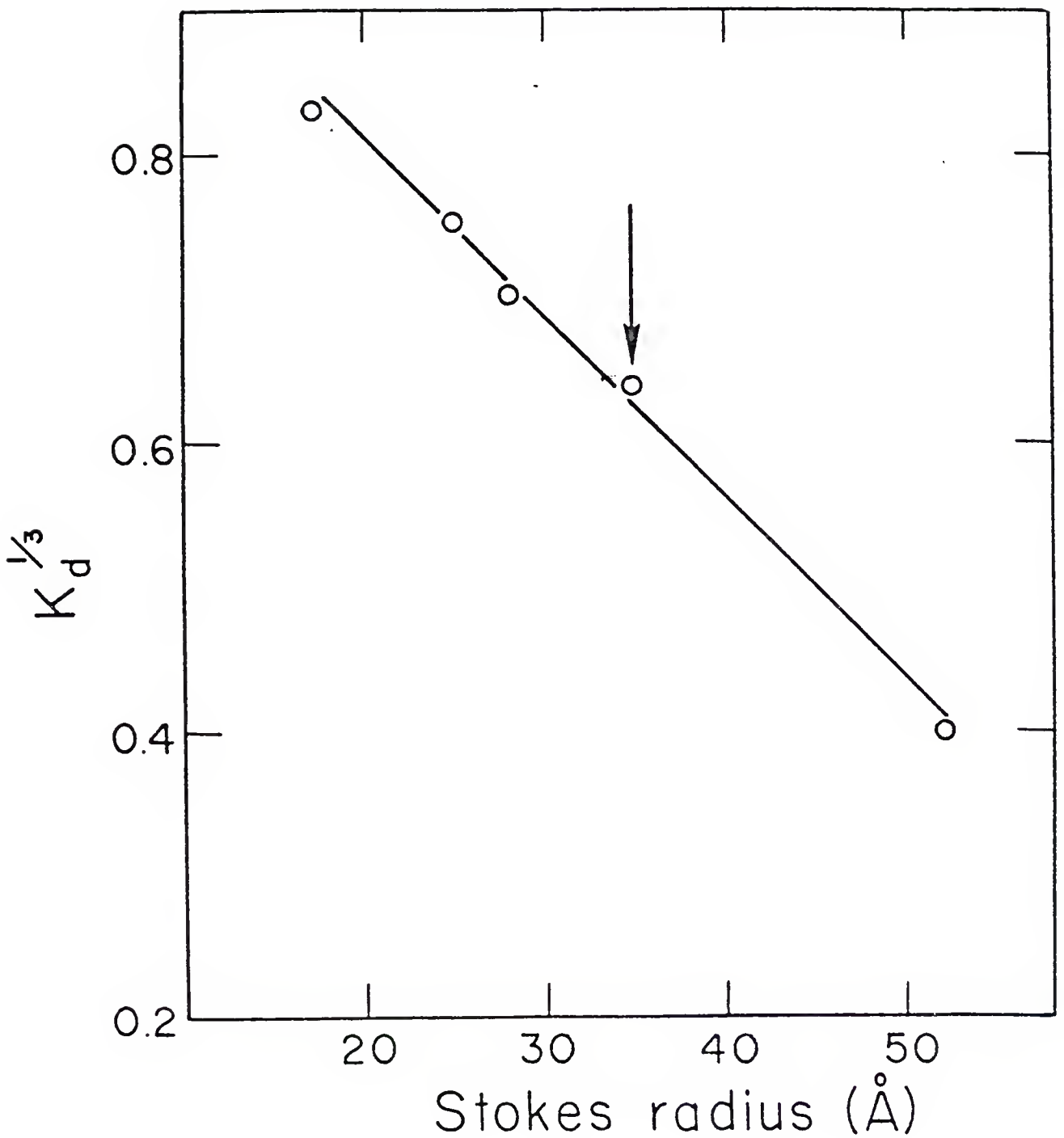

0.8
0.6
0.4
0.2
$K_d^{1/3}$
20
30
40
50
Stokes radius (Å)

Figure 5

Isoelectric focusing of nuclease β. 100 µl of fraction VI was layered on acrylamide tube gels and electrophoresed as described in chapter II. Parallel gels were cut in 2.5 mm slices. Slices from one gel were soaked in H_2O and the pH of the eluate was determined (●). Alternatively gel slices were soaked in buffer overnight and assayed for single strand nuclease activity as described in Chapter II (o).

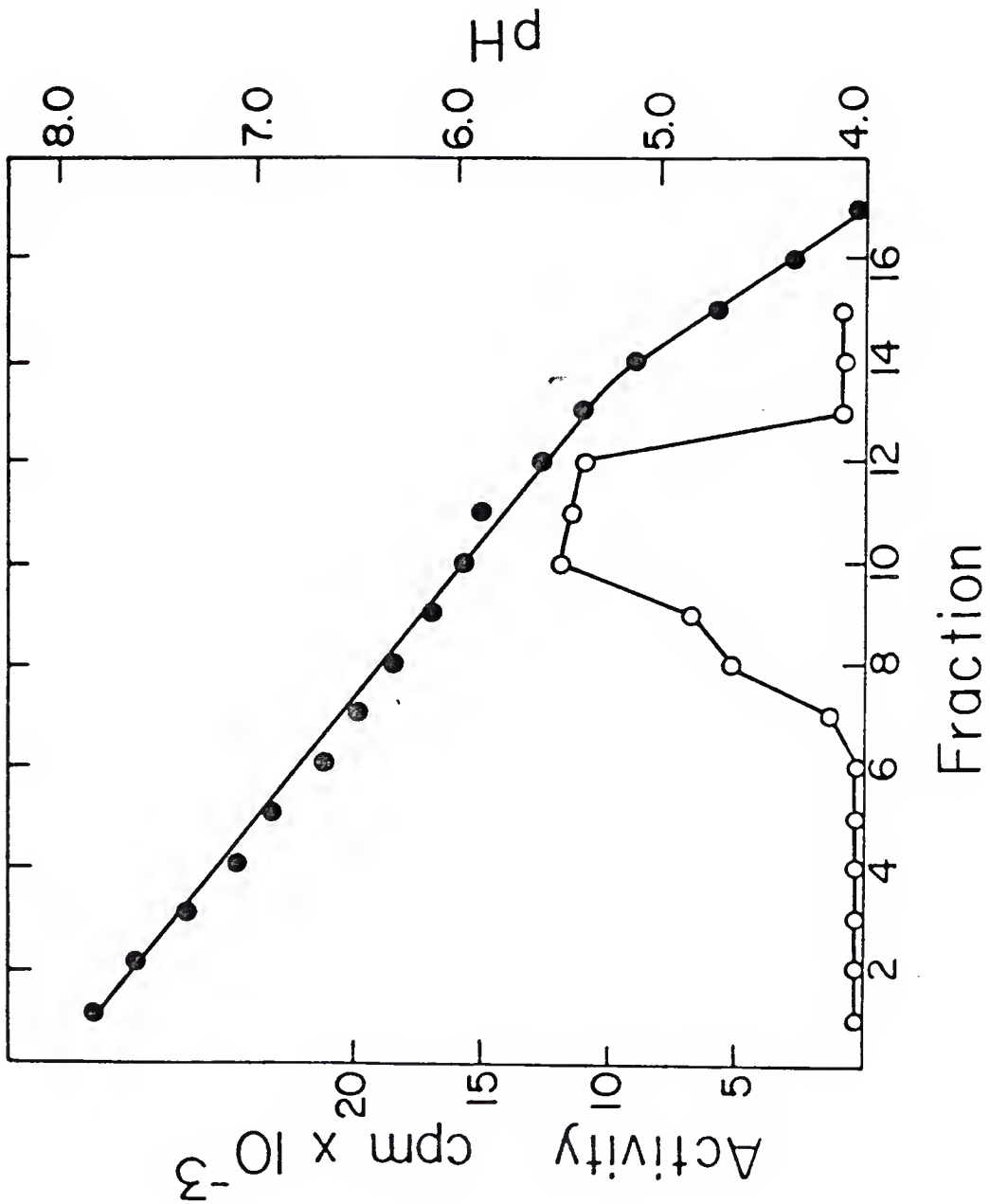

pH
8.0
7.0
6.0
5.0
4.0
Activity cpm x 10⁻³
5
10
15
20
Fraction
2
4
6
8
10
12
14
16

Figure 6

Time course of hydrolysis of DNA by nuclease β. Reaction mixtures (0.5 ml) containing 25 nmol of either denatured or native P22[^{3}H] DNA (2.5 X 10^{4} cpm per nmol) in 0.1 M potassium acetate, pH 6.0, were incubated with 10 units of nuclease β. At the indicated times aliquots of 0.1 ml were removed and the amount of DNA rendered acid soluble was determined.

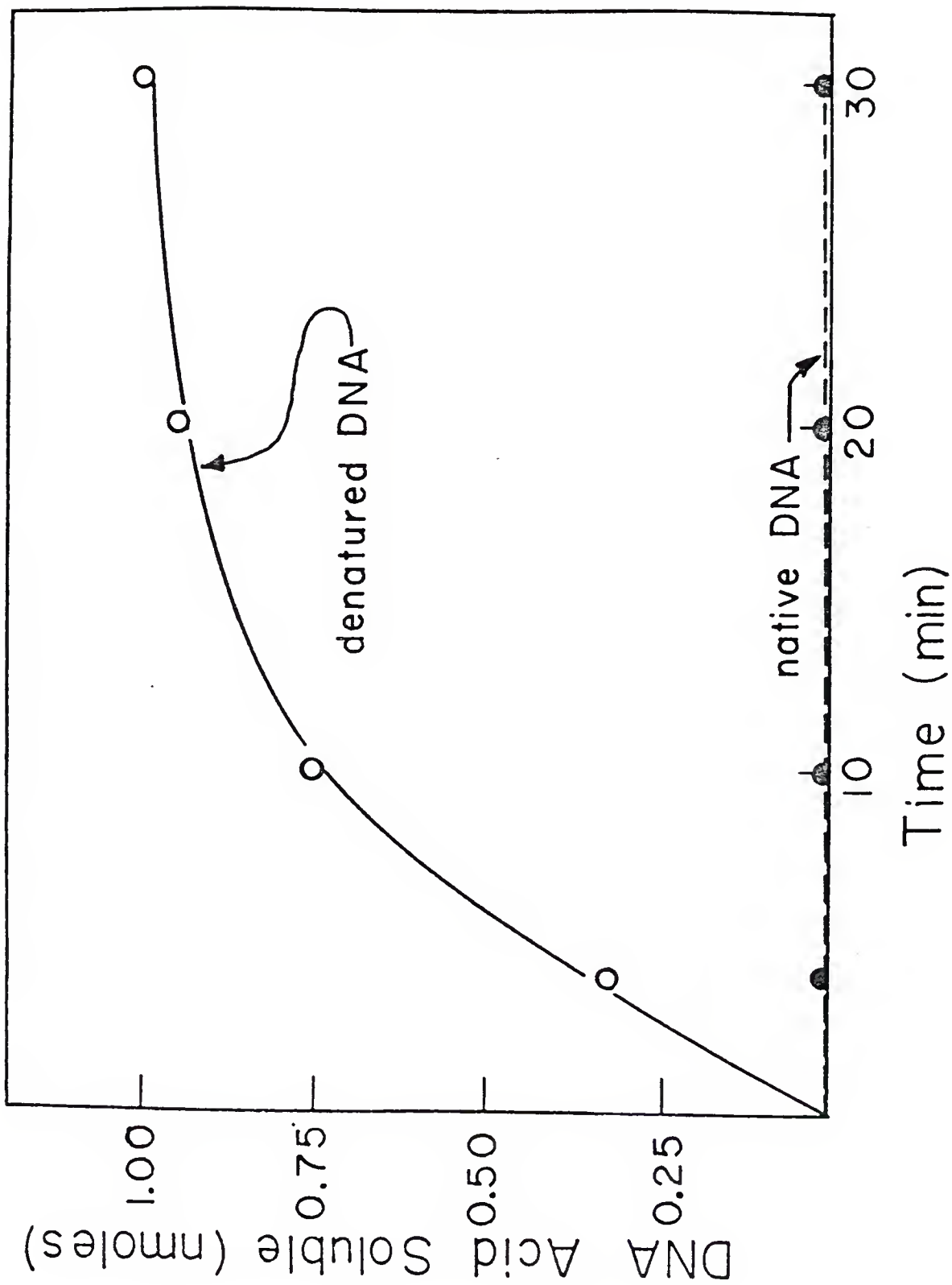

DNA Acid Soluble (nmoles)
1.00
0.75
0.50
0.25
denatured DNA
native DNA
10
20
30
Time (min)

Figure 7

Effect of pH on nuclease β activity. Reaction mixtures (0.1 ml) contained 3 nmol of denatured P22[^{3}H] DNA (2 X 10^{4} cpm/nmol) and 50 mM of the following buffer at the pH indicated. (Δ) K acetate; (X) Na citrate; (⊖) 2 (N-morpholino) ethane sulfonate; (o) sodium piperazine-N,N'-bis [2 ethane sulfonic acid] (▲) imidazole-HCl. Reactions were incubated for 10 min. at 37°C with 2 units of fraction VI and nuclease activity was measured as described in Chapter II. Activity at pH 6.0 in acetate buffer was taken as 100%.

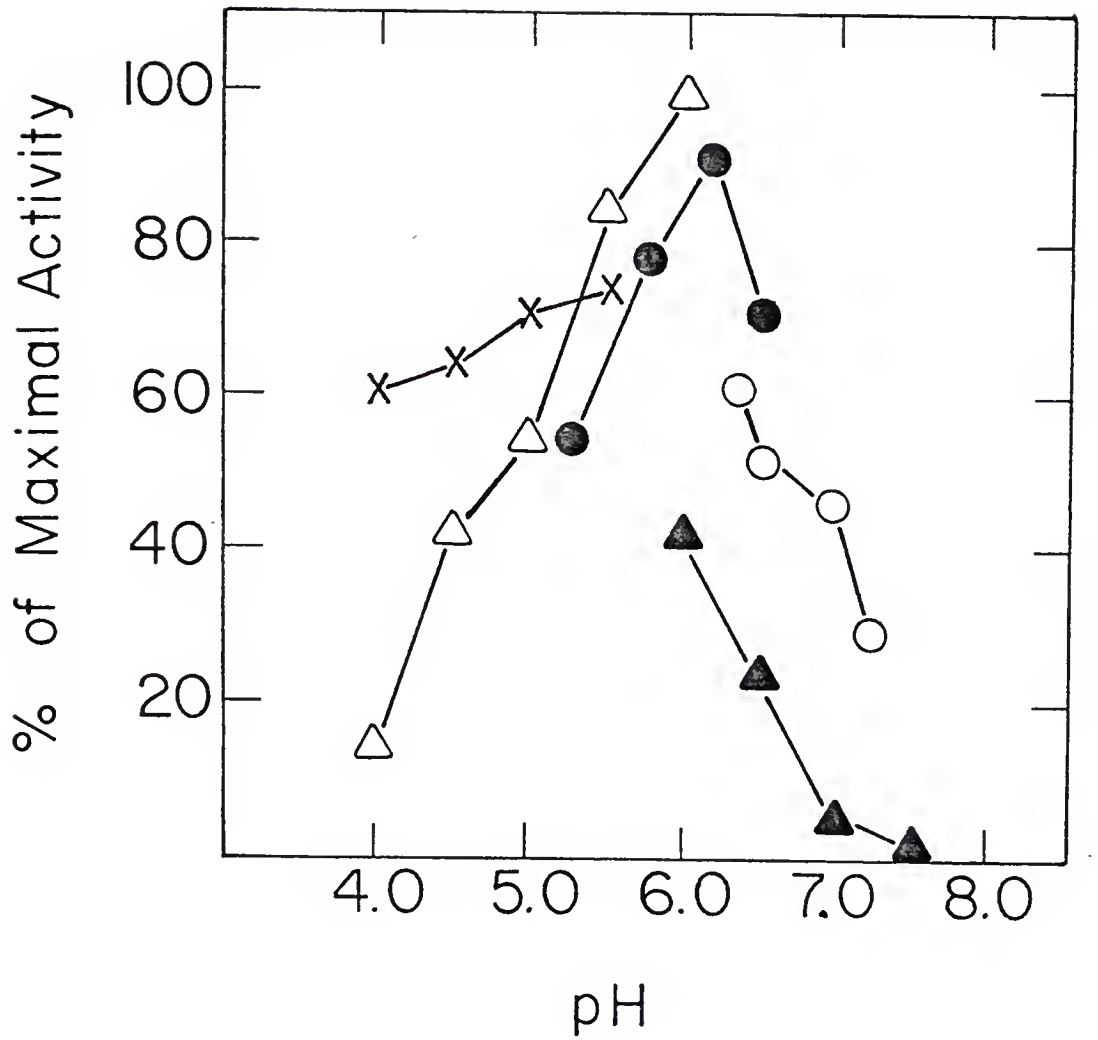
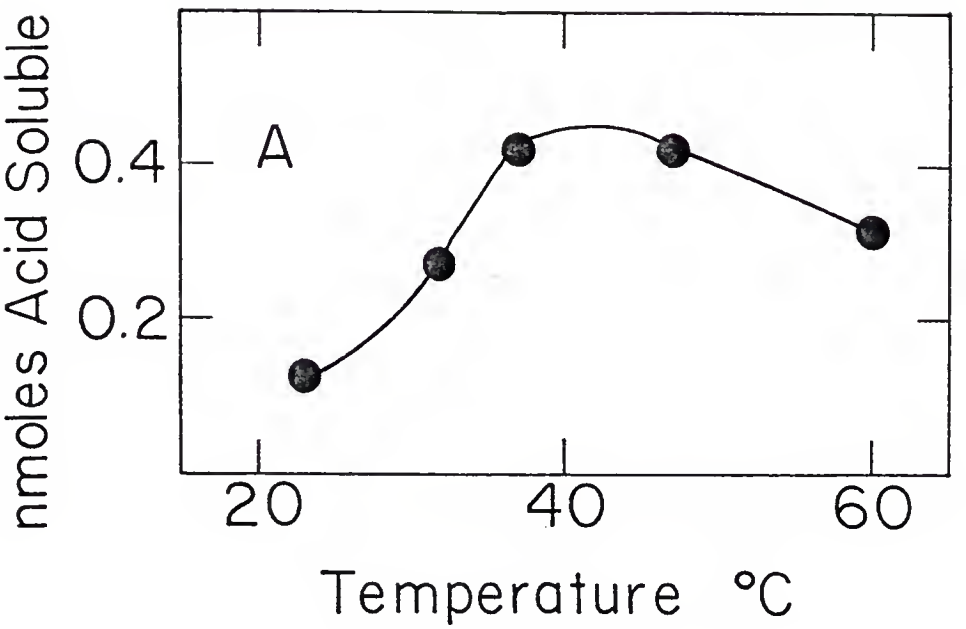
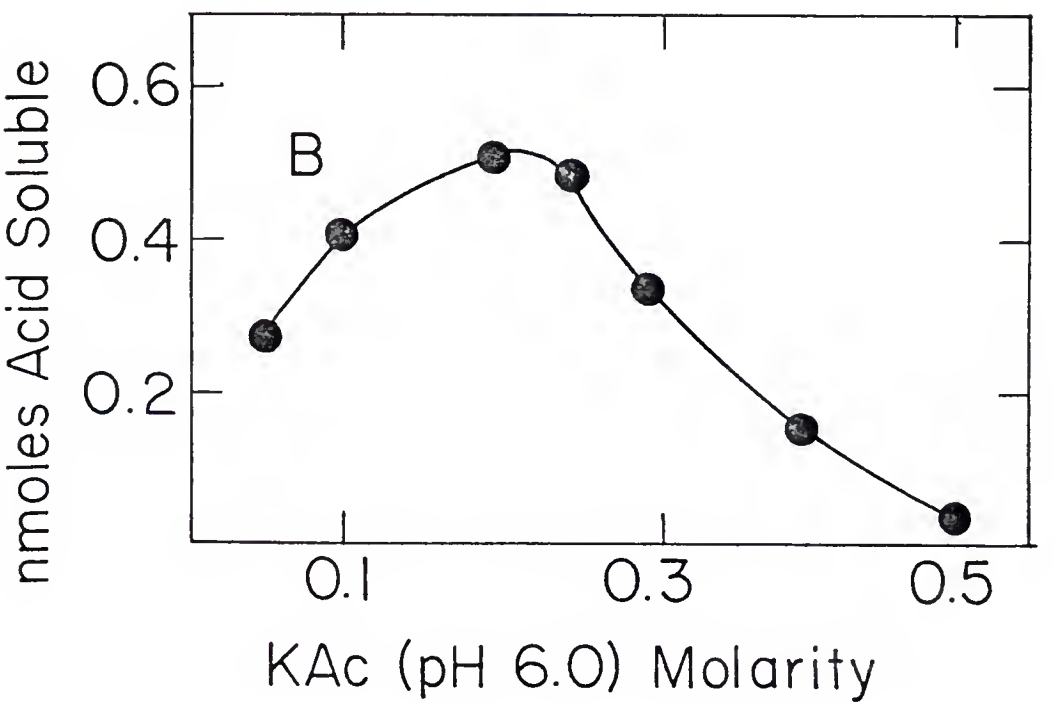

% of Maximal Activity
100
80
60
40
20
4.0
5.0
6.0
7.0
8.0
pH

Figure 8

Effect of temperature and ionic strength on nuclease β activity. A. Reaction mixtures (0.1 ml) containing 2 nmol denatured P22[^{3}H] DNA (2 X 10^{4} cpm/nmol) and 0.1 M potassium acetate were equilibrated at the temperature indicated. Three units of fraction VI nuclease were added and after 10 min. the amount of acid soluble DNA was determined, B. Reaction mixtures (0.1 ml) containing 2 nmol of denatured P22 [^{3}H] DNA and the indicated concentration of potassium acetate buffer were incubated for 10 min. at 37°C with 3 units of fraction VI nuclease. Acid soluble DNA was determined as described previously.


nmoles Acid Soluble
0.4
0.2
A
20
40
60
Temperature °C


nmoles Acid Soluble
0.6
0.4
0.2
B
0.1
0.3
0.5
KAc (pH 6.0) Molarity

contained 0.15 M sodium or potassium chloride. Hydrolysis of DNA by nuclease β was not affected by divalent cations (eg. Mg^{++}, Ca^{++}, Co^{++}, Mn^{++}) and was not inhibited by the chelating reagent ethylenediamine tetra-acetic acid (EDTA). Sulfhydryl reducing agents such as β-mercaptoethanol and dithiothreitol did not alter enzyme activity. N-ethylmaleimide, a sulfhydryl blocking reagent, did not affect nuclease β activity.

Inhibitors of Nuclease β

Nucleotides are potent inhibitors of nuclease β. ATP or dATP at 1 μM reduced nuclease activity 50% (figure 9). Phosphate and pyrophosphate were less effective inhibitors than the nucleoside deoxyadenosine. S 1 nuclease from Aspergillus oryzae is also strongly inhibited by nucleoside triphosphates (Wiegand et al., 1975). Unlike nuclease β, S 1 nuclease is more sensitive to phosphate than to deoxyadenosine.

Mode of Hydrolysis

Exonuclease Activity

Does nuclease β initiate hydrolysis from the ends of DNA molecules? Linear, uniformly labeled [^{3}H] DNA was labeled at the 5' or 3' end with ^{32}P (see Chapter II for preparation of substrate). A selective release of the ^{32}P-label to an acid soluble form would indicate terminal directed hydrolysis. When [^{3}H] DNA labeled at the 5'-end with ^{32}P was the the substrate for nuclease β the terminal ^{32}P became acid soluble faster than the ^{3}H label (figure 10A). When the nuclease

Figure 9

Effect of small molecules on nuclease β activity. Reaction mixtures (0.1 ml) containing 0.1 M potassium acetate, 2 nmole of denatured [^{3}H] P22 DNA and the indicated concentration of inhibitor were incubated for 10 min. at 37°C. The amount of DNA acid soluble was determined as described in Chapter II. (o) ATP or dATP; (●) AMP; (△) deoxyadenosine; (▲) phosphate; (■) pyrophosphate.

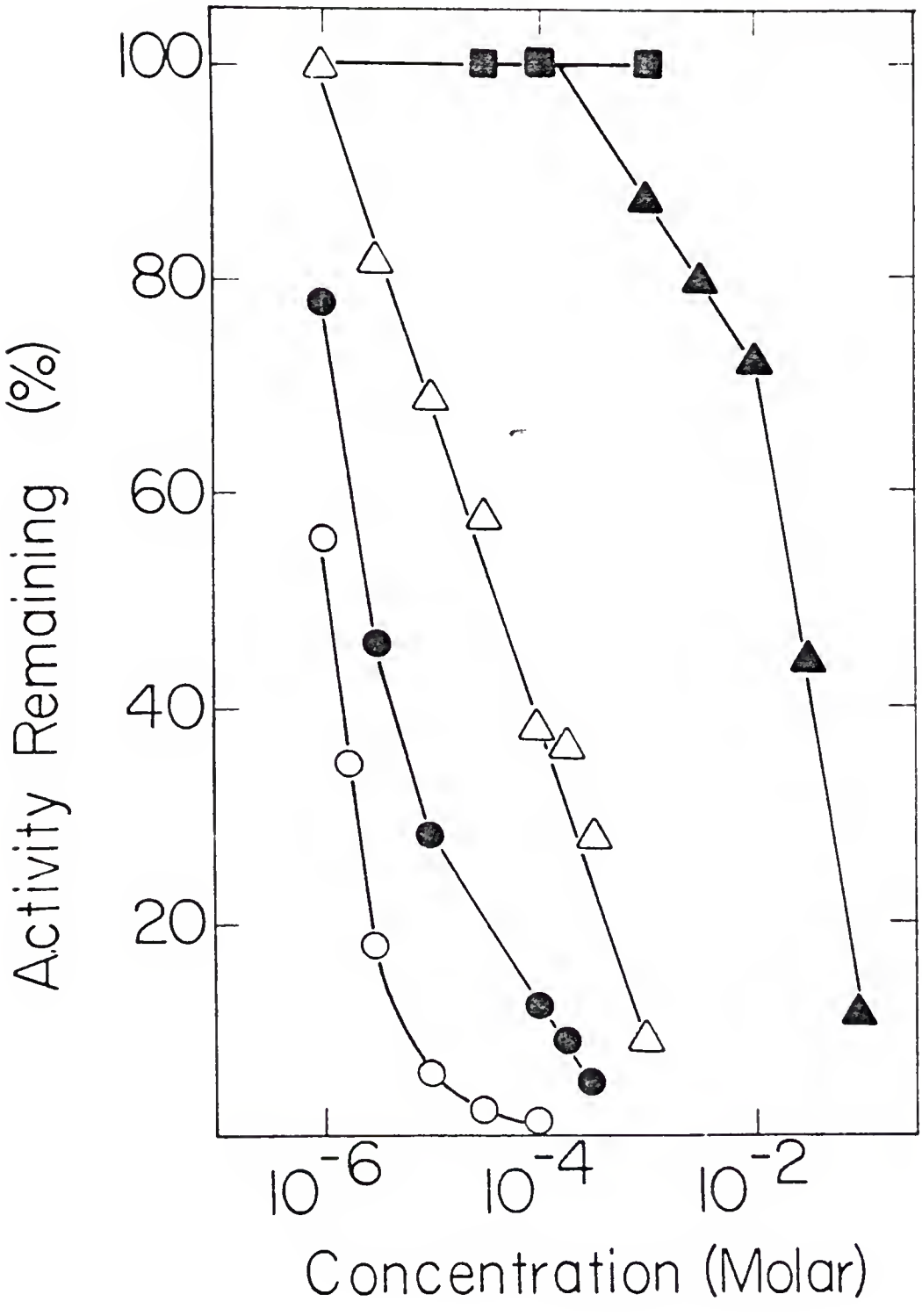
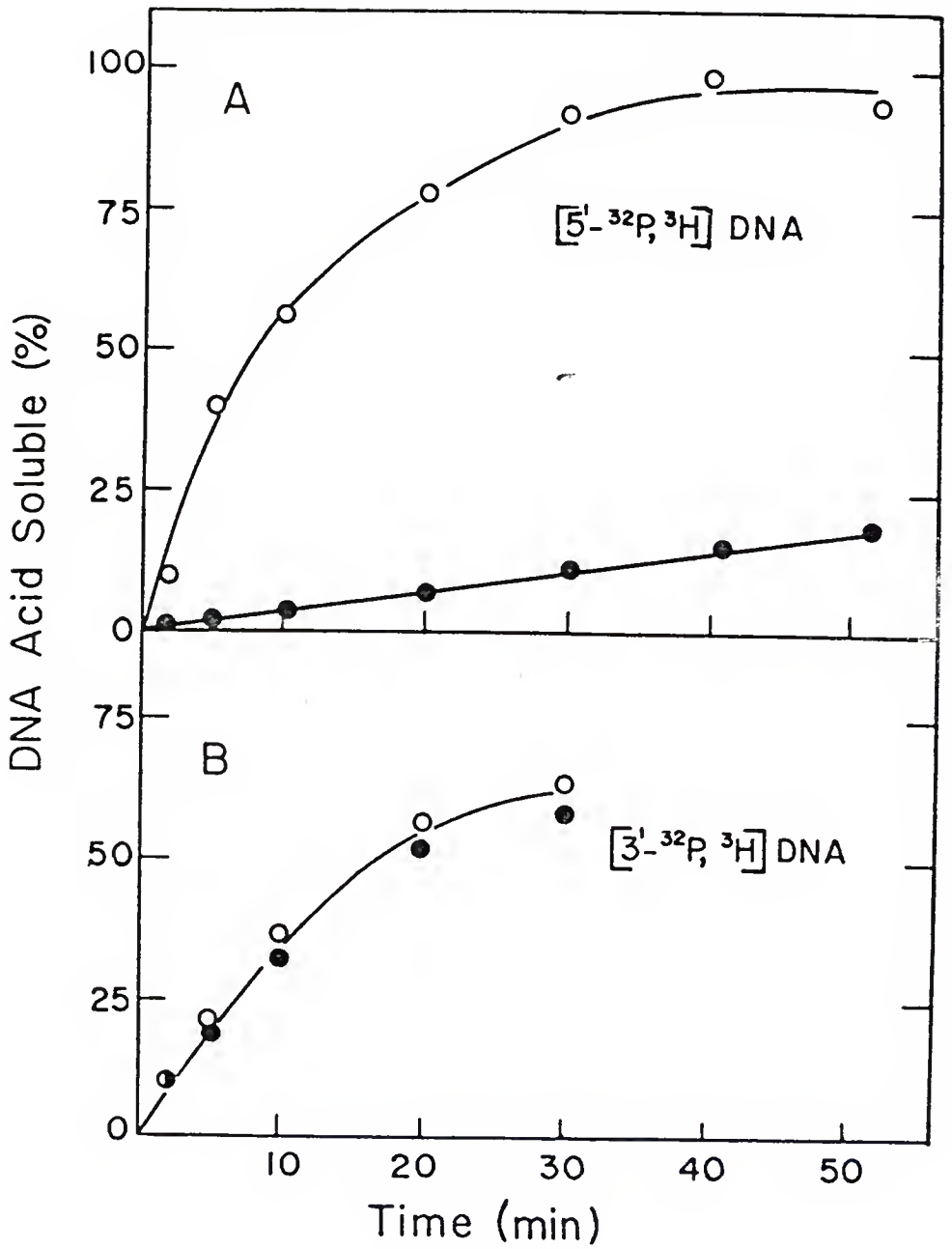

100
80
60
40
20
Activity Remaining (%)
10^{-6}
10^{-4}
10^{-2}
Concentration (Molar)

Figure 10

The action of nuclease β on terminally labelled DNA. A. A reaction mixture (1.0 ml) containing 16 nmol heat denatured øXRFIII[5'-^{32}P, ^{3}H] DNA (3.8 X 10^{3} cpm ^{32}P-label per nmol, 1.7 X 10^{4} cpm ^{3}H-label per nmol) in 0.1 M potassium acetate pH 6.0 was incubated with 10 units of nuclease β. At the indicated times aliquots (0.1 ml) were removed and the amount of label made acid soluble was determined. B. A reaction mixture (0.6 ml) containing 36 nmol heat denatured øXRFIII[3'-^{32}P, ^{3}H] DNA (1.2 X 10^{2} cpm ^{32}P-label per nmol, 4.2 X 10^{4} cpm ^{3}H-label per nmol) was incubated with 60 units of nuclease β and 0.1 ml aliquots processed as above. (o, ^{3}H), (●, ^{32}P).


100
75
50
25
0
A
[5'-32P, 3H] DNA
75
50
25
0
B
[3'-32P, 3H] DNA
10
20
30
40
50
Time (min)
DNA Acid Soluble (%)

substrate was [^{3}H] DNA ^{32}P-labeled at the 3'-end, both ^{32}P ^{3}H became acid soluble at the same rate (figure 10B). Preferential release of a terminal label suggests that nuclease β can hydrolyze DNA in an exonucleolytic fashion. This terminal hydrolysis is directed from the 5'-end of the polynucleotide.

Distributive Mode of Hydrolysis

I wanted to determine if nuclease β hydrolyzed a DNA molecule processively or if the enzyme dissociated from the substrate after each nucleolytic cleavage. Nuclease β was incubated with denatured ϕX174 [^{3}H] DNA at a 20-fold molar excess of DNA molecules to enzyme molecules. The moles of nuclease were determined by using a molecular weight of 68,000 and assuming the preparation was 70% pure nuclease. After 10 minutes an equal amount of unlabeled DNA was added to the reaction mixture (figure 11). Immediately the rate at which [^{3}H] DNA became acid soluble decreased 2-fold which was the same rate seen if the unlabeled DNA was present from the start of the reaction. Therefore nuclease β appears to hydrolyze DNA in a random or distributive fashion.

Activity on Circular DNA

Linear DNA appears to be hydrolyzed by nuclease β in an exonucleolytic manner. Does nuclease β require a free end to hydrolyze DNA? The enzyme was incubated with ϕX174 phage [^{3}H] DNA (a single stranded circular molecule) and the reaction products were sedimented through an alkaline sucrose gradient (figure 12A). The DNA sedimented more slowly after enzyme treatment suggesting extensive digestion of the circular

Figure 11

Distributive mode of DNA hydrolysis by nuclease β. A reaction mixture (1. 2 ml) containing 50 μM denatured ØXRFIII[^{3}H] DNA was incubated with 12 units of nuclease β and aliquots (0.1 ml) were removed for determination of the amount of digestion (●). At the time indicated by the arrow the reaction was split into two portions and the DNA concentration in one portion was increased to 100 μM by addition of unlabeled denatured ØXRFIII DNA (o). In a control tube equal amounts of ^{3}H-labeled and unlabeled DNA were mixed to give an initial DNA concentration of 100 μM. The reaction (0.5 ml) was started by addition of 5 units of enzyme and continued as above (Δ).

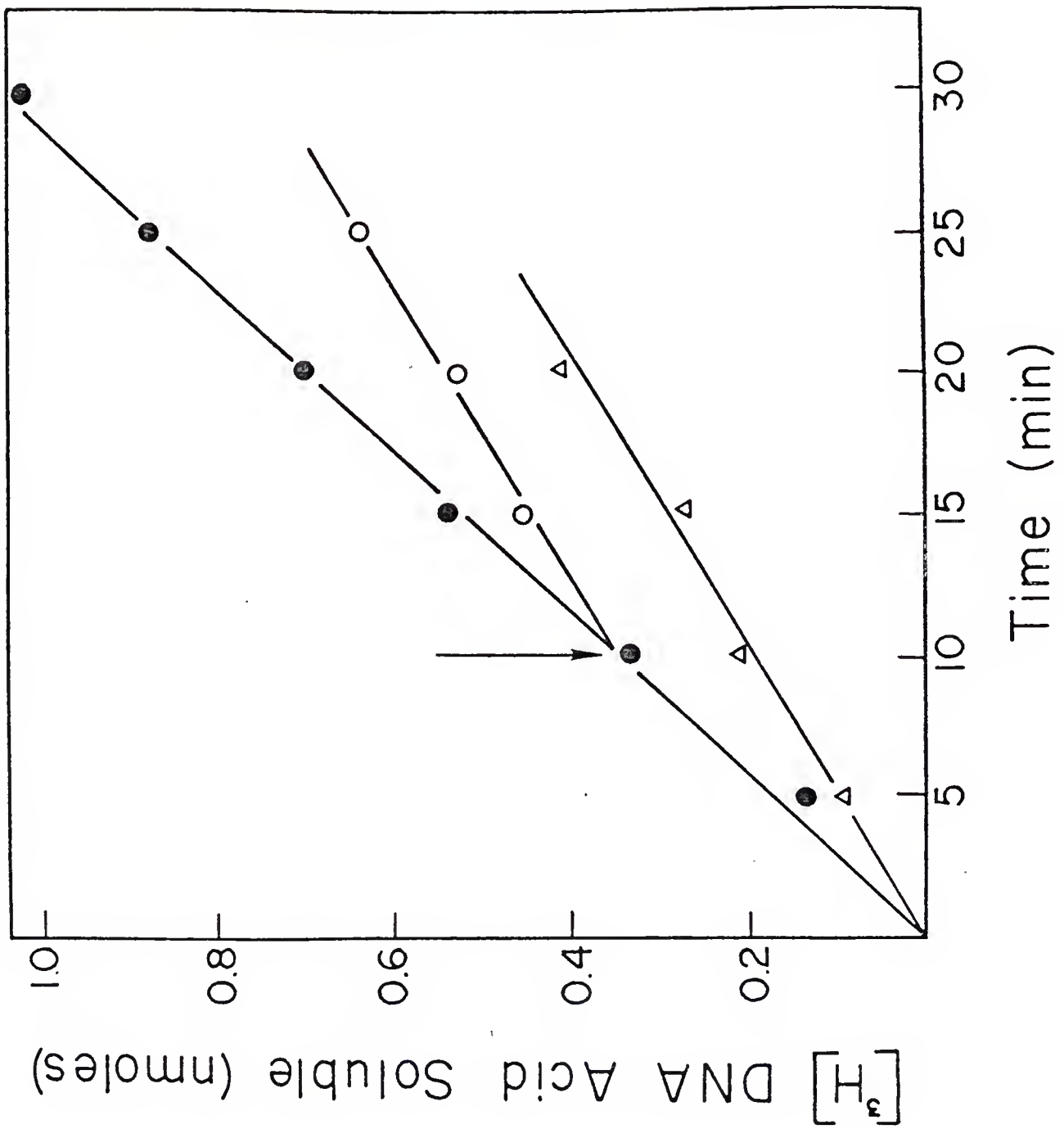
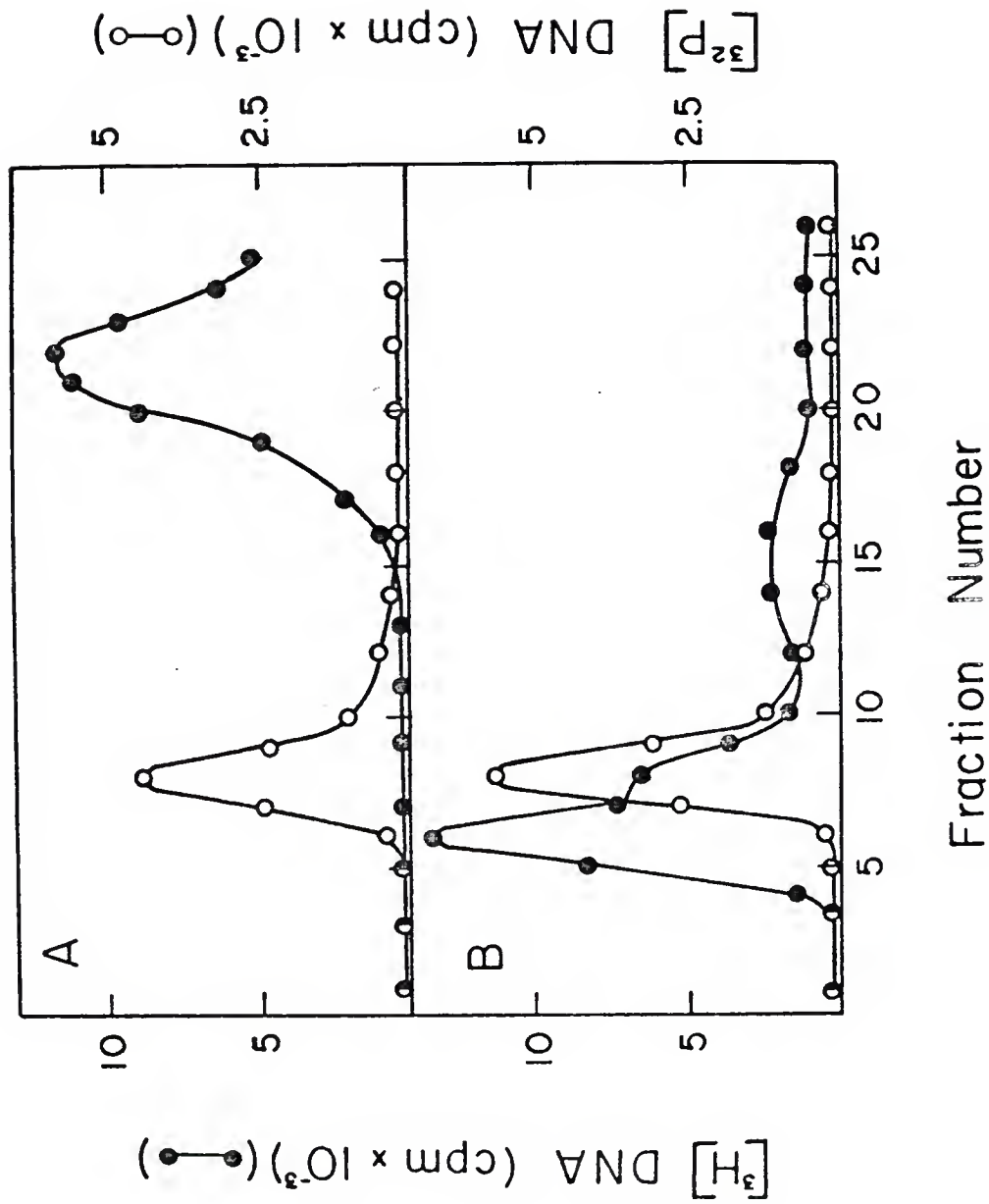

[³H] DNA Acid Soluble (nmoles)
0.2
0.4
0.6
0.8
1.0
5
10
15
20
25
30
Time (min)

Figure 12

Sucrose gradient sedimentation of øX174 phage DNA after treatment with nuclease β. Reaction mixtures (0. 1 ml) containing 2 nmol øX174 [^{32}P] DNA (1.7 X 10^4 cpm per nmol) and either 2.5 units of nuclease β (A.) or no enzyme (B.) were incubated at 37°C for 30 min. The reactions were stopped by addition of 10 µl 0.25 M EDTA and 30 µl 1 M NaOH and then 1.5 nmole øXRFIII [^{3}H] DNA (2.0 X 10^4 cpm per nmol) were added. The mixtures were layered on top an alkaline sucrose gradient and centrifuged 5 hr at 50,000 rpm in the Beckman SW50.1 rotor. Sedimentation is from right to left.


[3H] DNA (cpm x 10-3) (●—●)
[32P] DNA (cpm x 10-3) (○—○)
A
B
10
5
2.5
5
10
15
20
25
Fraction Number

substrate. This experiment suggests nuclease β can cleave DNA in an endonucleolytic manner.

Nuclease β can also nick form I DNA. Endonuclease activity was followed using an assay that measures the conversion of covalently closed, circular duplex DNA to a nicked form which can be selectively denatured at pH 12.3 and then retained on nitrocellulose filters (Kuhnlein et al., 1976). A comparison was made of the rate that nuclease β cleaves single or double strand DNA substrates. The endonuclease assay measured cleavage of form I DNA. Cleavage of single stranded [^{3}H] DNA was monitored by measuring the amount of ^{32}P susceptible to bacterial alkaline phosphatase (Kushner and Grossman, 1971). Nuclease β cleaves single stranded DNA 1,200-fold faster than form I DNA (table II). I believe both exonucleolytic and endonucleolytic hydrolysis are catalyzed by the same enzyme because these activities coelectrophoresed (figure 1) and cosedimented (figure 3).

Products of Hydrolysis

Size of Nucleotide Products

Denatured [^{3}H] DNA was made totally acid soluble by nuclease β. The products of hydrolysis were analyzed by chromotography on DEAE-cellulose in the presence of 7 M urea (Tomlinson and Tener, 1963). About 95% of the [^{3}H] label eluted at the size of mono- or dinucleotides (figure 13). When only 5% of the input [^{3}H] DNA was acid soluble 30% of the product was mononucleotide. Short oligonucleotides (chain length 2-7) contained between 5 and 15% of the acid soluble radioactivity. The formation of mononucleotide

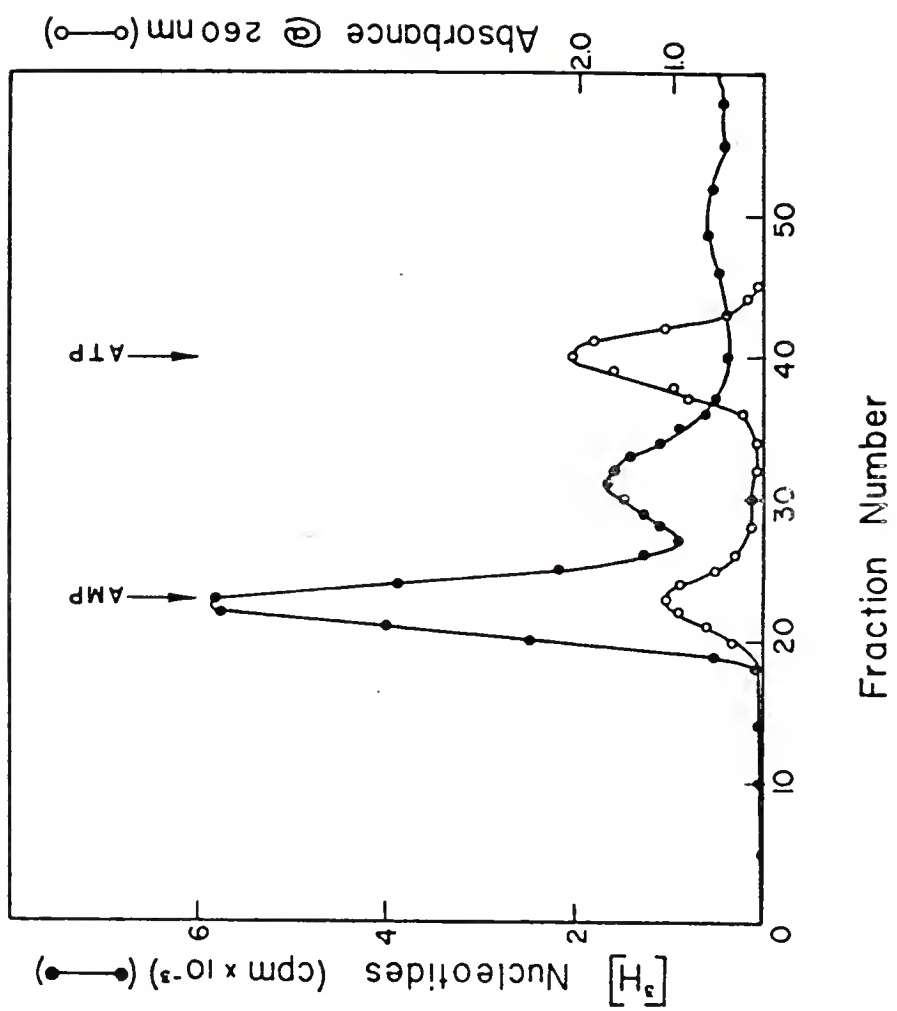
TABLE II. Reaction Rates of Nuclease β

DNA	Average chain length	% RF bound to filters	Bonds broken
			pmol
Single stranded	8.7	-	180
Superhelical	-	26.7	0.15

A reaction mixture of 0.1 ml containing 1.6 nmol of øX174 RFI[^{3}H] DNA (4.5 X 10^3 cpm/nmol) was incubated at 37°C with 1 unit of nuclease β. After 10 min, the number of molecules nicked was determined as describe in Chapter II. In an identical reaction, 1.6 nmol of single-stranded øX174[^{3}H] DNA (7.6 X 10^4 cpm/nmol) were substituted for the RFI DNA. After incubation for 10 min, the reaction was stopped by raising the pH to 8.0 with Tris-HCl and $MgCl_2$ was added to 10 mM. Alkaline phsophatase (0.25 unit) was added and the solution incubated at 45°C for an additional 10 min. Radioactivity unable to adsorb to norite was determined as described in Chapter II. The reciprocal of the fraction of ^{32}P label not adsorbing to norite was taken as the average chain length.

Figure 13

Size determination of the products of reaction. Denatured P22 [^{3}H] DNA (3 nmol at 2.5 X 10^4 cpm per nmol) was rendered 100% acid soluble by incubation for 1 h. with 60 units of nuclease β. The reaction mixture was applied to a DEAE-cellulose column (7 ml bed volume) equilibrated with 10 mM Tris-HCl pH 7.5, followed by addition of about 0.4 µmol AMP and 0.8 µmol ATP. The column was washed with 100 ml of 10 mM Tris-HCl pH 7.5, 7 M urea, then eluted with a 50 ml linear gradient from 0 to 0.3 M NaCl in the same buffer. Fractions of 0.9 ml were collected and 0.5 ml monitored for radioactivity and absorbance at 260 nm.


Absorbance @ 260 nm (o—o)
2.0
1.0
[³H] Nucleotides (cpm x 10⁻³) (●—●)
9
4
2
0
ATP
AMP
10
20
30
40
50
Fraction Number

residues as the major product of hydrolysis suggests nuclease β cleaves a single stranded substrate exonucleolytically.

Phosphoryl Ester Species of Digestion Products

Mononucleotides produced by nuclease β might contain either 5' or 3' phosphates. Two methods were used to analyze the mononucleotide products of digestion. [^{32}P] DNA was made 87% acid soluble by nuclease β. The products of digestion were treated with snake venom 5'-Nucleotidase, alkaline phosphatase, or nuclease P_1 (an enzyme with 3'-phosphomonoesterase activity). Phosphatase activity was measured by determining the amount of ^{32}P-label not adsorbed to norite (table III). Norite nonadsorbable ^{32}P was formed by alkaline phosphatase and nuclease P_1 but not by treatment with 5'-Nucleotidase. This indicates that nuclease β produces nucleoside 3'-monophosphates. Another way to analyze the phosphoryl ester species of mononucleotides is by chromatography on a Dowex column (Cohn and Volkin, 1951). This column can chromatographically separate 3' and 5' isomers of mononucleotides. In an experiment performed in collaboration with Dr. William Holloman, [^{32}P] mononucleotides produced by nuclease β co-chromatographed with nucleoside 3'-monophosphates included as standards (figure 14). Although the isomers of dTMP and dGMP used as standards did not totally separate it is clear the ^{32}P-label is skewed toward the trailing edge of the A_{260} peaks as would be expected for the 3' isomer.

The experiments described above show that mononucleotides produced by nuclease β contained 3' phosphates. Do

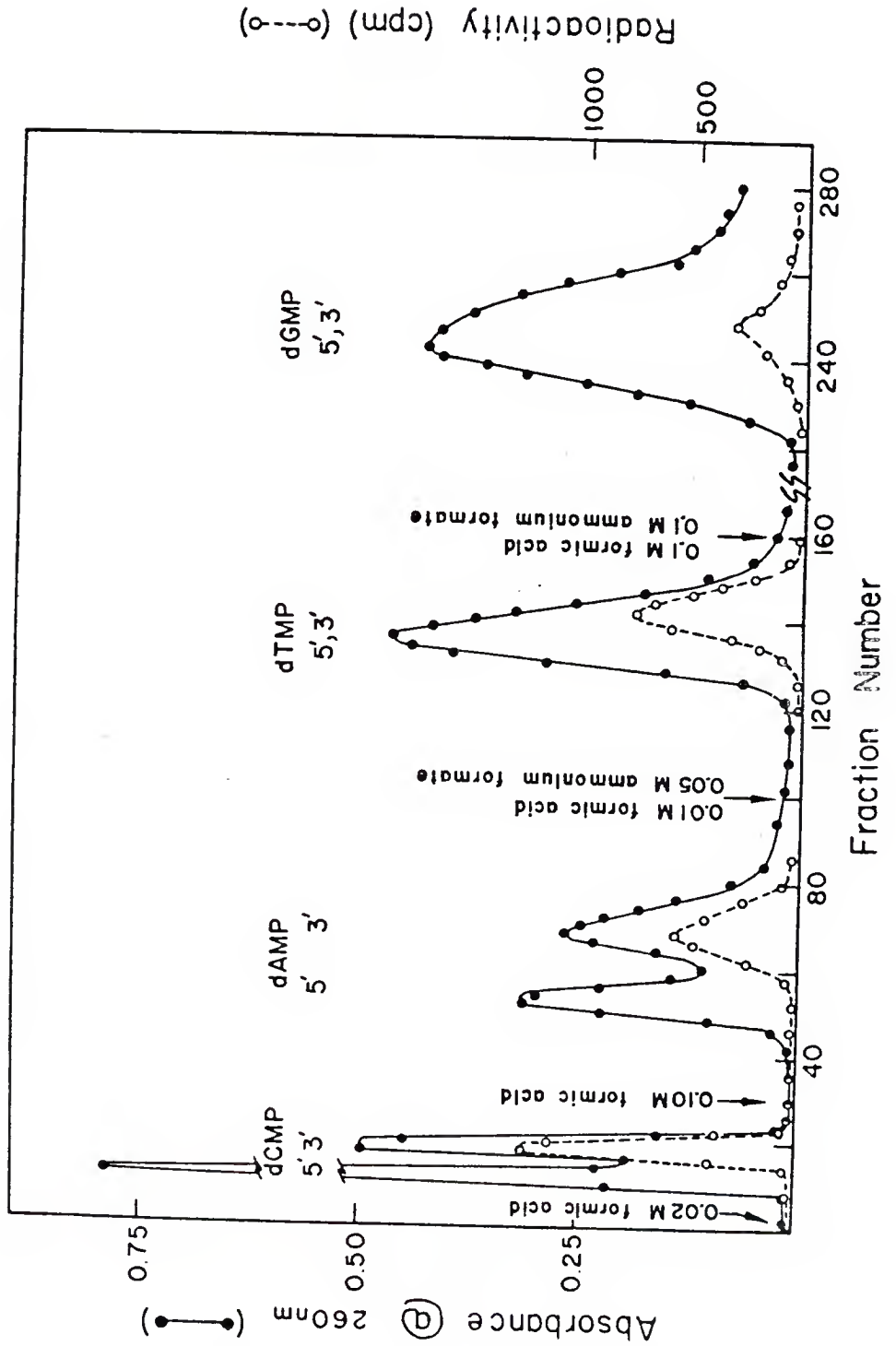
TABLE III. Mononucleotide Isomers of Digestion Products

Initial digest	Second digest	nmol [^{32}P]-norite nonadsorbable
Nuclease β (87% acid soluble)	5'-Nucleotidase	0.019
	Alkaline phosphatase	1.19
	Nuclease P1	3.04
	None	0.06

A solution (0.4 ml) containing 50 mM potassium acetate, pH 5.5, and 18.4 nmol of øX174[^{32}P] DNA (3.2 X 10^4 cmp/nmol) was incubated at 37°C with 40 units of nuclease β. After 30 min, the solution was made 10 mM in $MgCl_2$. Aliquots of 0.1 ml removed for further digestion with 5'-nucleotidase, alkaline phosphatase, or nuclease P1 were treated as follows: (a) 20 µl of 1.0 M sodium glycine, pH 9.0 and 1 unit of 5'-nucleotidase; (b) 20 µl of 1.0 M Tris-HCl, pH 8.0, and 0.05 unit of alkaline phosphatase; and (c) 10 units of nuclease P1. Reaction mixutres were incubated for 20 min at 37°C and ^{32}P label unable to adsorb to norite was determined (see Chapter II).

Figure 14

Chromatography of 5'- and 3'-mononucleotides on Dowex-1. øX174 [^{32}P] DNA (4 nmol at 2.5 X 10^4 cpm per nmol) was digested to completion by incubation at 37°C for 2 hrs with 100 units of nuclease β. About 10 A_{260} units of each of the 5'- and 3'-isomers of the four deoxymonucleotides were added to the [^{32}P] DNA digest, the mixture diluted to 10 ml with H_2O and then loaded on a column (0.4 X 14 cm) of Dowex-1 (formate). The column was devloped with the buffers indicated (Cohn and Volkin, 1951). Fractions of 2.2 ml were collected. Radioactivity in 0.5 ml aliquots was determined by scintillation counting.


Absorbance @ 260nm (●——●)
Radioactivity (cpm) (o---o)
Fraction Number
0.02 M formic acid
0.10M formic acid
0.01 M formic acid
0.05 M ammonium formate
0.1 M formic acid
0.1 M ammonium formate
dCMP 5'3'
dAMP 5' 3'
dTMP 5',3'
dGMP 5',3'

oligonucleotides produced by nuclease β contain 3' phosphates and 5' hydroxyls at the chain termini? In collaboration with Dr. William Holloman, acid precipitable oligonucleotides produced by nuclease β were examined as substrates for snake venom phosphodiesterase (see Chapter II). The snake venom enzyme is a 3'-directed exonuclease that is more active upon oligonucleotides bearing 3' hydroxyls (Turner and Khorana, 1959). As seen in table IV, prior treatment of nuclease β generated oligonucleotides with alkaline phosphatase increased the activity of snake venom phosphodiesterase ten-fold. This experiment suggests the oligonucleotides produced by nuclease β are terminated by 3' phosphates.

Other Activities

5'-Nucleotidase Activity

The most purified preparations of nuclease β could remove a phosphate from 5' mononucleotides. ^{32}P-labeled mononucleotides prepared by digestion of [^{32}P] DNA with nuclease P_1 was a substrate for nuclease β preparations and snake venom 5'-nucleotidase (table V). Routine 5' nucleotidase assays were performed using mononucleotides generated by sequential digestion of [^{32}P] DNA with pancreatic DNAse followed by snake venom phosphodiesterase (see Chapter II). The 5' nucleotidase activity coelectrophoresed (figure 1) and cosedimented (figure 3) with the activity hydrolyzing single stranded DNA. The reaction optima (eg. pH, ionic strength and activity in the presence of EDTA) for nucleotidase activity were identical to those for hydrolysis of denatured DNA. Nuclease β does not

TABLE IV. Activity of Snake Venom Phosphodiesterase on DNA Partially Digested by Nuclease β

Nuclease β DNA digest	Rate of hydrolysis by phosphodiesterase
	nmol/min
Untreated	0.095
Dephosphorylated	1.04

øX174 [^{3}H] DNA was made 23% acid soluble by treatment with nuclease β. The acid precipitable DNA was recovered and half the DNA was treated with alkaline phosphatase. Subsequent digestion with phosphodiesterase was performed as described in Chapter II.

TABLE V. 5'-Nucleotidase Activity of Nuclease β

Initial digest	Second digest	nmol [^{32}P]-norite nonadsorbable
Nuclease P1 (97% acid soluble)	5'-Nucleotidase	3.44
	Alakaline phasphatase	3.58
	Nuclease β	2.72
	None	0.12

A 0.4-ml solution containing 50 mM potasssium acetate, pH 5.5, 10 mM $MgCl_2$ and 18.4 nmol of [^{32}P] DNA (3.2 X 10^4 cpm/nmol) was incubated for 30 min with 40 units of nuclease P1. Aliquots of 0.1 ml removed for further digestion with 5'-nucleotidase, alkaline phosphatase, or nuclease β were treated as follows: (a) 20 µl of 1.0 M sodium glycine, pH 9.0, and 1 unit of 5'-nucleotidase; (b) 20 µl of 1.0 M Tris-HCl, pH 8.0, and 0.05 unit of alkaline phosphatase; and (c) 10 units of nuclease β. These aliquots were incubated for 20 min at 37°C and ^{32}P label unable to adsorb to norite was determined.

produce free phosphate from p-nitrophenol phosphate, a synthetic compound that is a substate for alkaline phosphatase. I could not detect any ATP hydrolysis (less than 5 pmol) when incubating [γ-^{32}P]ATP with enough nuclease β to hydrolyze 25 nmol of denatured DNA.

I suggested earlier that nuclease β hydrolyzed linear DNA in a 5'-directed manner because ^{32}P-label was preferentially released from [^{3}H] DNA labeled at its 5'-end with [^{32}P]PO_4. Perhaps nuclease β is not an exonuclease and the release of ^{32}P was an artifact of 5' nucleotidase activity. To distinguish between these possibilities 5'-[^{32}P] DNA was digested with nuclease β and the amount of label made acid soluble or norite nonadsorbable was determined (figure 15). ^{32}P-label was made acid soluble at a higher rate than the formation of free ^{32}P suggesting that nuclease β is indeed a 5'-directed exonuclease.

Activity on RNA

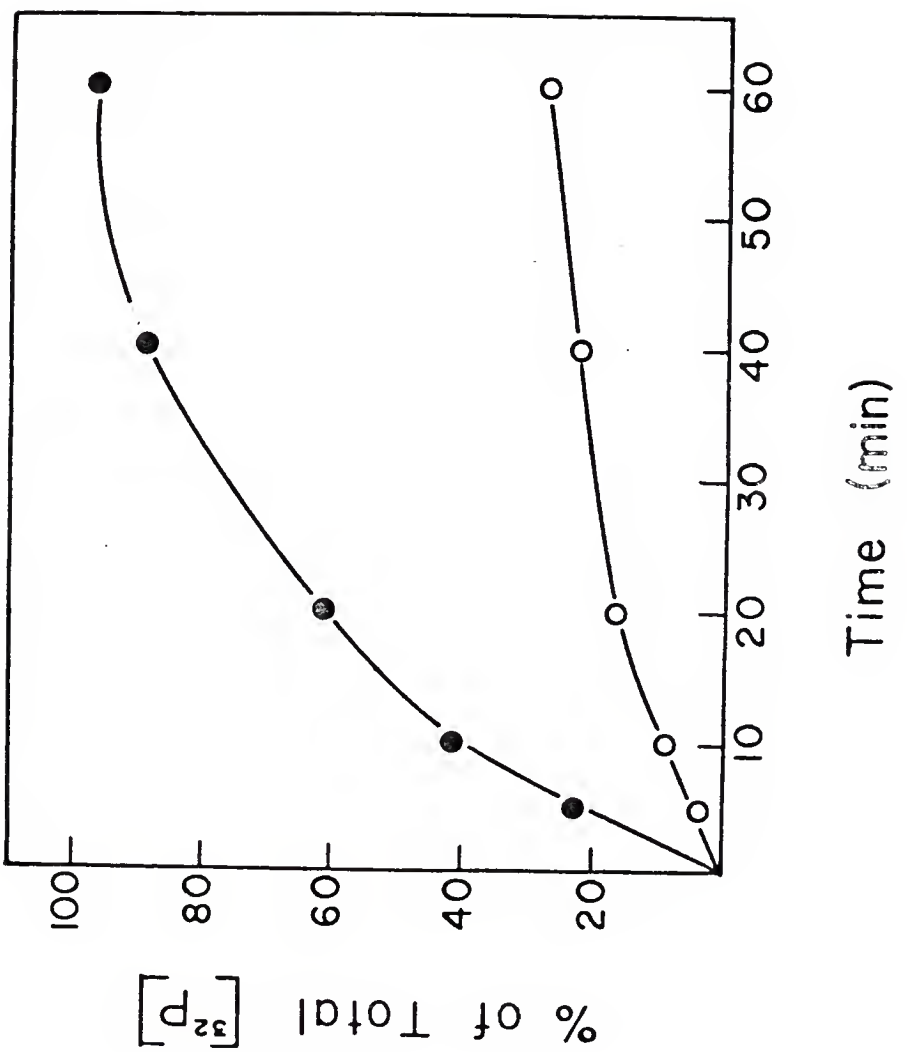
Hela cell ribosomal RNA was used to determine if nuclease β can hydrolyze RNA. Electrophoresis of the reaction products in acrylamide tube gels showed the RNA migrated farther in the gel than untreated RNA. The smaller molecular weight RNA produced was of a discrete size suggesting either site specific cleavage of the RNA or some secondary structure in ribosomal RNA prevented further digestion. It cannot be ruled out that RNAse activity is due to a contaminant in nuclease β fractions.

Summary

Nuclease β, isolated from wild type cells of Ustilago maydis, is a single polypeptide of 68,000 d that is highly

Figure 15

Identification of the initial product of reaction with 5'-terminally labeled DNA. A reaction mixture (1.2 ml) containing 72 nmol denatured P22 [5'-^{32}P] DNA (2.7 X 10^4 cpm per nmol) and 36 units of nuclease β was incubated at 37°C. At the indicated times two 0.1 ml aliquots were removed for determination of acid soluble radioactivity (●) and for determination of the amount of ^{32}P-label that no longer adsorbed to norite (o).


% of Total [32P]
100
80
60
40
20
10
20
30
40
50
60
Time (min)

active on single stranded DNA. The enzyme digests linear DNA in a 5'-directed exonucleolytic manner producing mostly mononucleotide products. The nuclease can endonucleolytically cleave circular DNA and both mononucleotide and oligonucleotide products contain a 3' phosphate. The enzyme also possesses 5' nucleotidase activity and may hydrolyze RNA.

CHAPTER IV
PURIFICATION AND CHARACTERIZATION OF DBP III

DNA is thought to exist in a cell as a highly folded, topologically constrained molecule (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Ide et al., 1975; Laemmli et al., 1977). Proteins and RNA appear to be responsible for holding eucaryotic DNA folded in supercoiled loops (Georgiev et al., 1978; Benyajata and Worcel, 1976; Cook et al., 1976). Superhelicity alters the secondary structure of DNA and this change can be recognized by some proteins that bind DNA. For example, histone H1 demonstrates a binding preference for DNA that is supercoiled (Vogel and Singer, 1975; Singer and Singer, 1978). In the experiments described below a search was designed to examine extracts of U. maydis for proteins binding to superhelical DNA. Three chromatographically separable activities were found that bind to superhelical DNA. The first part of this chapter discusses the purification of DNA binding activities and presents some properties of a nuclease activity copurifying with one of the DNA binding activities. The latter part of this chapter describes the characterization of a nuclease - free DNA binding activity, termed DBP III.

Purification

The assay used to purify DNA binding activity is a modification of the filter assay originally developed by Riggs et al. (1970) (see Chapter II). Protein and radioactive

form I DNA are mixed and filtered through a nitrocellulose filter which traps DNA-protein complexes but does not retain protein-free DNA. The reaction sample (0.1 ml) was diluted to 1 ml with 5 X SSC and filtered. The filters were washed with two 1 ml aliquots of 5 X SSC, dried and counted for radioactivity. Approximately 5% of the DNA bound without protein addition and this amount could not be reduced by further washing of the filter.

A detailed description of the steps used in purification of DNA binding activities is given in chapter II. Nucleic acid free extracts were found to be difficult to assay reproducibly until the protein was further fractionated by DEAE-cellulose chromatography. It was necessary to include EDTA in all assays until the final step in purification to prevent nicking of the substrate by endogenous nucleases. Nucleic acid free extracts were applied to a DEAE-cellulose column and absorbed proteins were eluted with an increasing gradient of NaCl. Fractions containing binding activity were dialyzed to remove the NaCl and applied to a phosphocellulose column and eluted with a NaCl gradient. The activity that bound to the phosphocellulose column was concentrated by precipitation with ammonium sulfate and layered onto a Sephacryl S-300 column. Two peaks of activity were observed to elute from this column. The first peak eluted after 120 ml of buffer was collected and the second peak eluted after 180 ml of buffer had passed through the column.

Most (90%) of the activity in Fraction III was not retained when the extract was passed through a phosphocellulose column (figure 16A, peak 1). The DNA-binding activity that did not absorb to phosphocellulose was applied to a Sephacryl S-300 column. DNA binding activity eluted slightly ahead of BSA which was run as a standard. Fractions demonstrating DNA binding activity from this Sephacryl column contained at least ten polypeptides when electrophoresed in a SDS-gel (see chapter II). The major staining band migrated as a 60,000 d polypeptide when compared to the mobility of standard proteins. Since the DNA binding activity not absorbing to phosphocellulose was found not to bind to a variety of other columns (eg. hydroxylapatite, carboxymethyl cellulose and Affigel-Blue), the characterization of DNA binding was not pursued.

The DNA binding activity absorbing to phosphocellulose separated into two distinct peaks when applied to a Sephacryl S-300 column. Approximately 70% of the binding activity applied to the Sephacryl column eluted at 180 ml slightly behind a BSA standard (figure 16B, peak 2). The fractions containing DNA binding activity also possessed a Mg^{++} stimulated nuclease activity (Table VI). The nuclease, active on single stranded DNA, had a pH optimum of 8.0 (figure 17). Several factors suggested to me that this nuclease activity may be due to the presence of U. maydis nuclease α (Holloman et al., 1981). Both nuclease α and the nuclease eluting from Sephacryl are highly active on single stranded DNA, have a pH

Figure 16

Chromatographic separation of DNA binding activity. A. Phosphocellulose chromatography of fraction III DNA binding activity as described in Chapter II. Peak 1 represents DNA binding activity that did not absorb to phosphocellulose. Peak 2 represents DNA binding activity (fraction IV) eluting from 0.1 to 0.15 M NaCl. (●) DNA binding activity was assayed as described in Chapter II. (o) Molarity of NaCl. B. Sephacryl S-300 filtration of fraction IV DNA binding activity as described in Chapter II. The arrows indicate the position of elution of standards blue dextran (BD) and bovine serum albumin (BSA). Fraction IV DNA binding activity was concentrated by precipitation with ammonium sulfate and layered on a Sephacryl S-300 column. Peak 1 represents DNA binding activity (o) that eluted in the same fractions as the standard, blue dextran (BD). This DNA binding activity is termed DBP III. Peak 2 represents DNA binding activity (●) and single stranded deoxyribonuclease activity (o) that coeluted behind the standard, BSA.

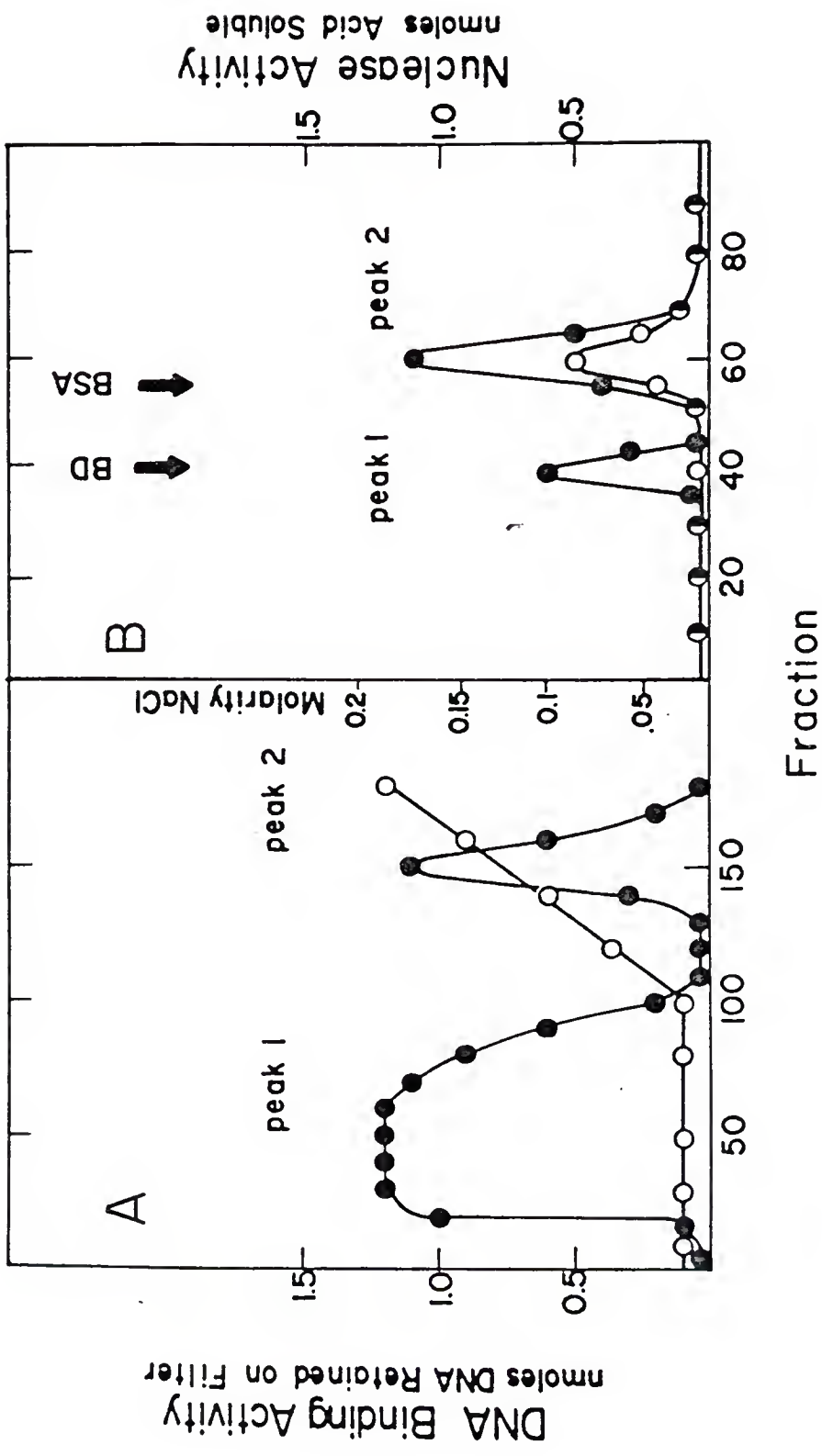

DNA Binding Activity
nmoles DNA Retained on Filter
A
peak 1
peak 2
1.5
1.0
0.5
50
100
150
Molarity NaCl
0.2
0.15
0.1
.05
B
BD
BSA
peak 1
peak 2
Nuclease Activity
nmoles Acid Soluble
1.5
1.0
0.5
20
40
60
80
Fraction

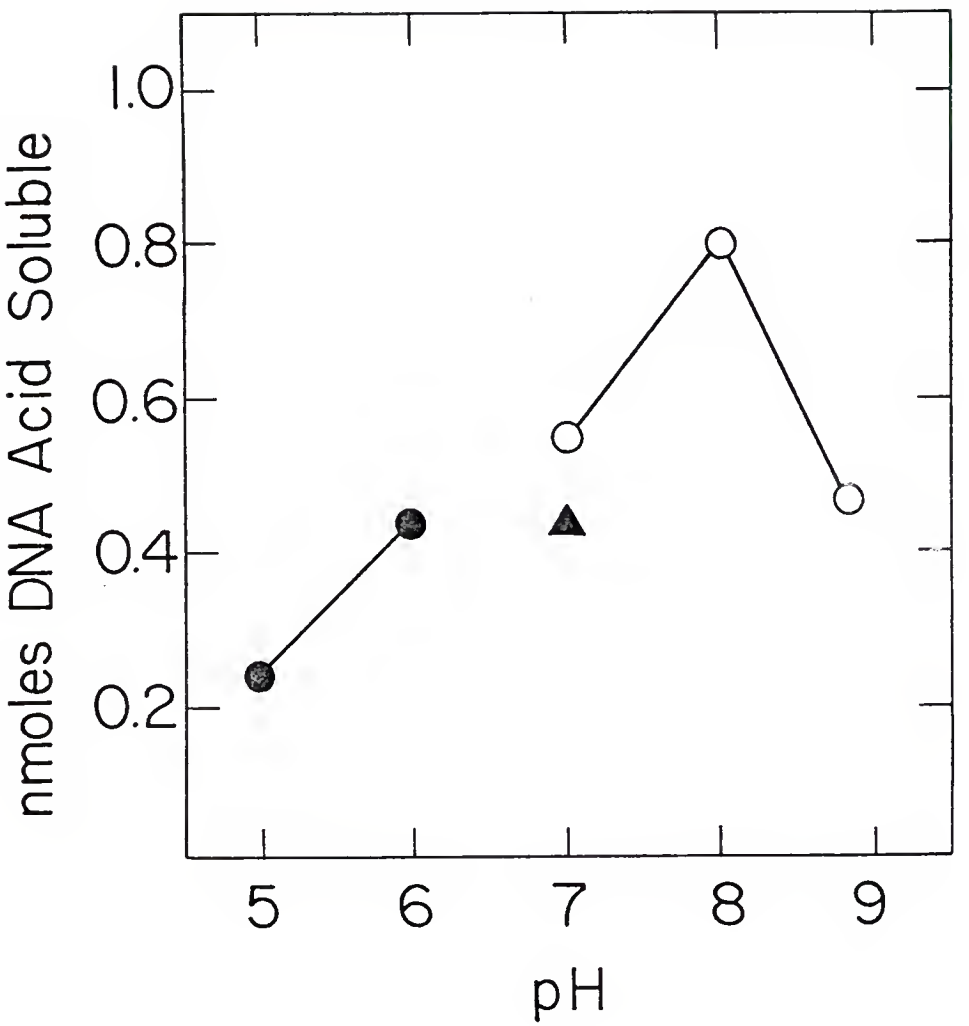
TABLE VI. Effect of Small Molecules on Nuclease Activity

	% Activity
Standard*	100
- Mg^{++}	10
- Mg^{++} + 2 mM $CaCl_2$	10
- Mg^{++} + 0.1 mM $CoCl_2$	90
+ 10 mM β-mercaptoethanol	80
+ 1 mM ATP	100
+ 10 mM EDTA	< 5

*Reaction mixtures (0.1 ml) containing 2 nmol of denatured P22 [^{3}H]DNA (2.5 X 10^4 cpm/nmol), 0.1 M Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and 10 µl of the Sephacryl S-300 eluate (fraction 58) were incubated 15 min at 37°. Acid soluble radioactivity was measured as described in Chapter II. 100% activity represents 0.2 nmole of DNA acid soluble.

Figure 17

pH optimum of nuclease activity. Reaction mixtures (0.1 ml) containing 2 nomol denatured P22 DNA (2.5 X 10^4 cpm/nmol), 5 mM $MgCl_2$ and 0.1 M of either potassium acetate (●), potassium phosphate (Δ) or Tris-HCl (o) were incubated with 10 µl of the Sephacryl eluate (fraction 59). After 15 min. at 37°C the amount of DNA made acid soluble was determined (see chapter II).


nmoles DNA Acid Soluble
1.0
0.8
0.6
0.4
0.2
5
6
7
8
9
pH

optimum of 8.0 and are inhibited by EDTA. Polypeptides from the Sephacryl column fractions containing nuclease activity were analyzed by SDS-gel electrophoresis. The major band had a mobility corresponding to a molecular weight of 55,000. The polypeptide in nuclease α preparations responsible for nuclease activity has a molecular weight of 55,000 (Holloman et al., 1981). Another way to compare nuclease α and the nuclease activity from the Sephacryl column was to assay purified nuclease α for DNA binding using the filter assay. I found that, in the presence of EDTA, purified nuclease α preparations retain form I DNA on nitrocellulose filters. In addition, the ratio of DNA binding activity to single strand nuclease activity was the same for two different preparations of nuclease α (Table VII). These data suggest to me that the fractions eluting from Sephacryl at 180 ml contain nuclease α.

The DNA binding activity eluting from the Sephacryl S-300 column at 120 ml (Fraction V) could be stored at 4°C for 2 months without loss of activity. The DNA binding activity, termed DBP III, was further characterized as described below.

Fraction V was subjected to SDS-gel electrophoresis (see chapter II). The major band had a molecular weight of 47,000 relative to protein standards. The amount of 47,000 d protein in the gel was approximated by comparing the intensity of Coomassie stain with standard proteins. Twenty percent of the protein applied to the gel (5 µg) could be visualized as a 47,000 d band. Why would a polypeptide of 47,000 d elute

TABLE VII. DNA Binding Activity in Purified Nuclease α Preparations

Nuclease α	Nuclease Activity	DNA Binding Activity
Preparation	units/ml	nmol DNA bound/ml
1	200	300
2	40	60

Two different preparations of Ustilago nuclease α were assayed for the ability to retain form I DNA to nitrocellulose filers. Purification of nuclease α and the determination of nuclease activity were performed by Dr. William Holloman as described previously (Holloman et al., 1981). Retention of form I DNA to nitrocellulose filters in the presence of 1.5 M NaCl was measured as described in Chapter II.

from Sephacryl columns at the position of blue dextran? Small polypeptides can elute from gel filtration columns as apparently large molecules for various reasons. For example, the 47,000 d polypeptide could be the reduced subunit of a larger protein. Perhaps protein aggregation by ionic or hydrophobic interactions could result in the observed elution profile from Sephacryl. Renaturation of the polypeptides separated by SDS-gel electrophoresis and reconstitution of DNA binding activity would be required to definitively state that the 47,000 d polypeptide is the DNA binding protein.

DBP III (Fraction V) was assayed for nuclease and topoisomerase activity. DBP III sufficient to bind 1 nmole of RFI DNA did not nick RFI DNA (less than 25 pmol) or hydrolyze single stranded DNA (less than 10 pmol). Although 1 nmole of RFI DNA was bound by the protein no detectable covalently closed relaxed DNA (RFIV) was observed when the sample was deproteinized and analyzed by agarose gel electrophoresis (less than 0.2 nmol).

Purified DBP III was free of nuclease activity and bound to form I DNA. I observed that DBP III did not retain form II DNA on nitrocellulose filters. This apparent specificity for form I DNA led me to characterize the interaction of DBP III with DNA.

Reaction Conditions for DBP III

Maximal binding activity of DBP III was observed at pH 6.0 in acetate buffer. At pH 7.5 in Tris-HCl buffer 20-fold less binding activity was seen as compared to pH 6.0 in

acetate (figure 18). Binding was not affected by including either divalent cations (eg. Mg^{++}, Ca^{++} or Co^{++}) or EDTA in the reaction mixture (Table VIII). Addition of reducing agents such as β-mercaptoethanol and dithiothreitol or addition of the sulfhydryl blocking reagent N-ethylmaleimide had no effect. High energy compounds such as ATP and NAD did not change the amount of DNA bound by DBP III. Addition of SDS or proteinase K to reaction mixure (0.5%) ablated DNA binding activity. Binding was very fast but showed a threshold temperature of 26°C (figure 19).

When reactions were performed in buffer containing 0.2 M NaCl a 50% reduction was seen in DNA binding activity. If the NaCl was added after incubating the reaction for 5 min. at 37°C there was no decrease in binding (Table VIII). The same amount of DNA binding activity was observed if the reaction mixture (0.1 ml) was diluted and filtered in 0.5 X SSC or in 10 X SSC. These results suggest that protein binding to DNA is sensitive to high ionic strength but once formed the protein - DNA complex is resistant to dissociation by salt. Although many nuclear proteins are removed from DNA by 2 M salt extraction some protein material remains bound to the chromosome and maintains the highly folded structure of DNA (Benyajata and Worcel, 1976; Adolf et al., 1977). Histones (Spelsberg and Hnilica, 1971) and helix destabilizing proteins (Herrick and Alberts, 1976) are examples of DNA binding proteins which are dissociated from DNA by 2 M salt.

Figure 18

Optimal pH of DBP III activity. DNA binidng activity was assayed as described in Chapter II. Reaction mixtures (total volume of 0.1) containing 2 nmol of form I øX174 [^{3}H] DNA and 0.1 M of either potassium acetate (●), sodium piperazine-N,N'-bis[2 ethane sulfonic acid] (o) or Tris-HCl (▲) at the indicated pH were incubated with 25 µl of Fraction V DBP III. Incubation was for 10 min. at 37°C.

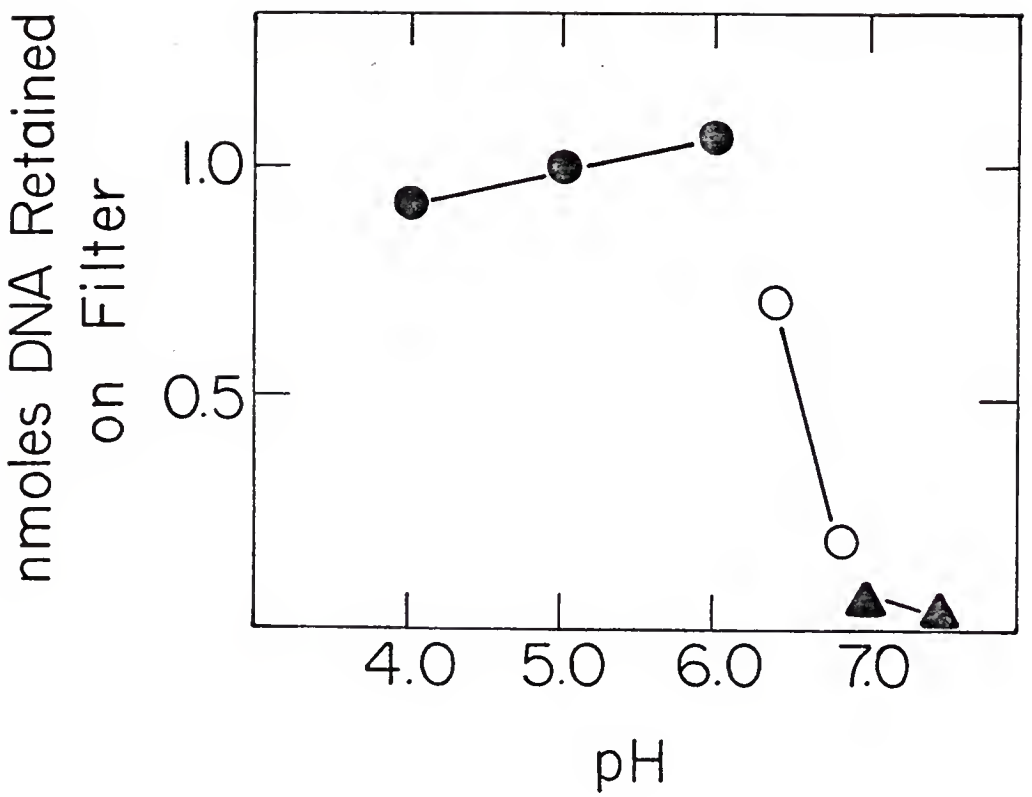

nmoles DNA Retained on Filter
1.0
0.5
4.0
5.0
6.0
7.0
pH

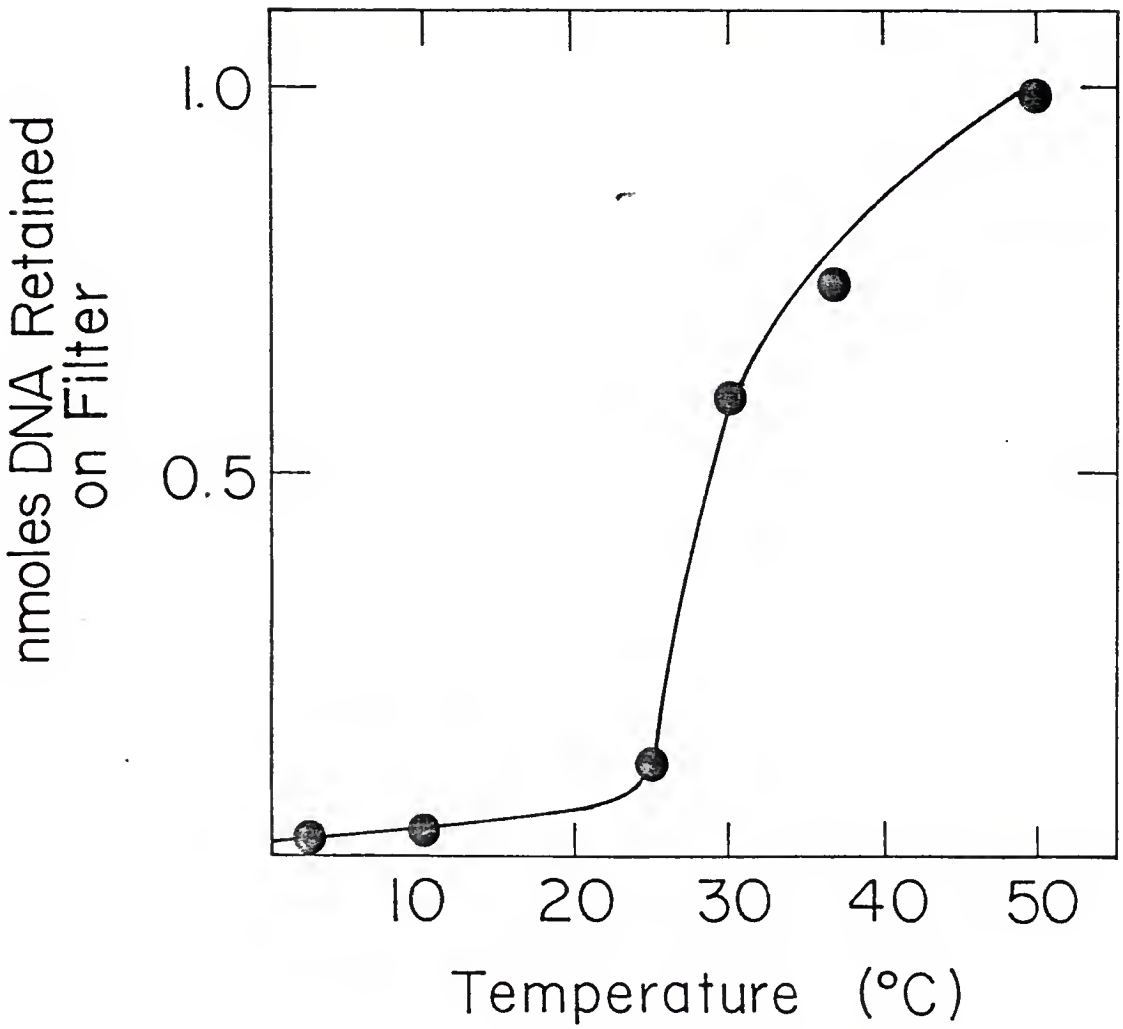
TABLE VIII. Effect of Small Molecules on DNA Binding

Reaction Conditions	% Activity
Standard*	100
+ 10 mM $MgCl_2$	100
+ 2 mM $CaCl_2$	100
+ 0.2 mM $CoCl_2$	100
+ 2 mM EDTA	100
+ 10 mM β-mercaptoethanol	100
+ 10 mM N-ethylmaleimide	100
+ 1 mM ATP	100
+ 0.2 M NaCl	50
+ 0.5% SDS	0
37° for 5 min then:	
+ 2 M NaCl	100
+ 0.5% SDS	0

*Reaction mixtures (0.1 ml) containing 0.1 M potassium acetate, 1 nmol [^{3}H]-labeled øX174 form I DNA and sufficient DBP III to bind 0.5 nmole of DNA were incubated at 37° for 10 min except where indicated. DNA binding activity was measured as described in Chapter II.

Figure 19

Effect of temperature on DBP III activity. Reaction mixtures (total volume of 0.1 ml) containing 1 nmole of øX174 [^{3}H] form I DNA and 0.1 M potassium acetate were incubated at the indicated temperatures. 20 µl of Fraction V DBP III was added and incubation was continued for 10 min. The reaction was terminated by addition of 1 ml of 5 X SSC and DNA binding activity was measured as described in Chapter II.


nmoles DNA Retained on Filter
1.0
0.5
10
20
30
40
50
Temperature (°C)

Characterization of Protein Binding to DNA

When increasing amounts of DBP III were incubated with form I DNA there was a linear increase in the quantity of DNA retained on the filter (figure 20). However, if the form I DNA was replaced by form II or form III DNA, there was no retention of DNA on the filter. DBP III dependent retention of form I DNA may indicate DBP III binds to covalently closed DNA. Form I DNA may be a substrate for DBP III because the DNA is superhelical. Alternatively, binding to form II or form III DNA may occur but the protein dissociates before the complexes are filtered. Reaction mixtures containing DBP III and a mixture of form I and II DNA can be electrophoresed in agarose gels. Samples containing DBP III showed an altered mobility of form I DNA (figure 21). The mobility of form II DNA was unaltered by addition of DBP III. The altered mobility of form I DNA was not observed if SDS (0.5%) was added to the reaction sample prior to electrophoresis.

The experiments described above led me to examine why form I DNA but not form II or form III DNA was retained on filters by DBP III. Is form I DNA a substrate for DBP III because it is superhelical or because the DNA molecule is covalently closed? The role of superhelicity in binding was examined by using DNA relaxed by topoisomerase (form IV DNA) as a substrate for DBP III.

Form IV DNA was prepared by treating form I DNA with Ustilago topoisomerase (see Chapter II). DBP III was incubated with either form I or form IV DNA and the samples were

Figure 20

Activity with form I, form II or form III DNA as substrate. The indicated amounts of fraction V DBP III were incubated in standard reaction mixures (see Chapter II) containing 1 nmole of either form I (o), form II (●) or form III (●) øX174 [^{3}H] DNA. Form II DNA was isolated by chromatographic separation of a mixture of form I and form II DNA using a column containing acridine yellow (see Chapter II). Form III DNA was made by cleavage of øX174 form I DNA with the single cut restriction enzyme Pst I (see Chapter II). DNA binding activity was determined as described previously.

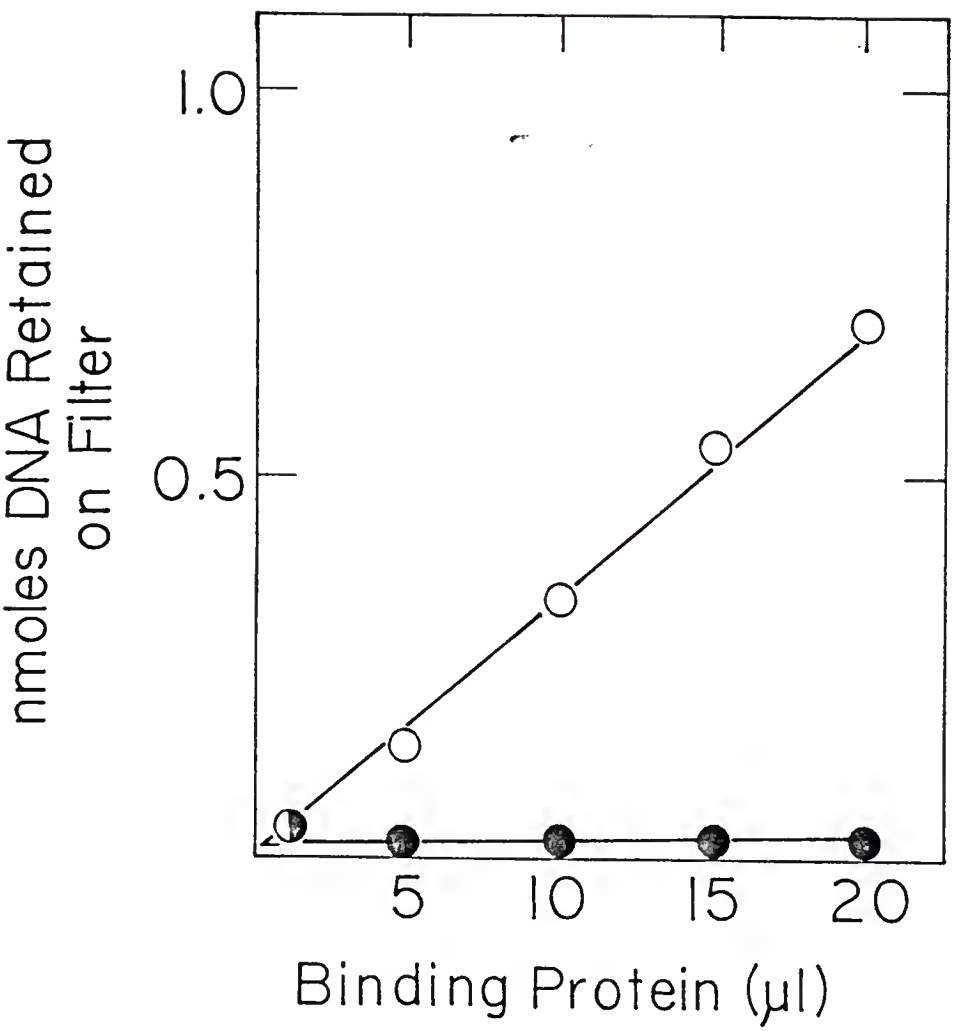
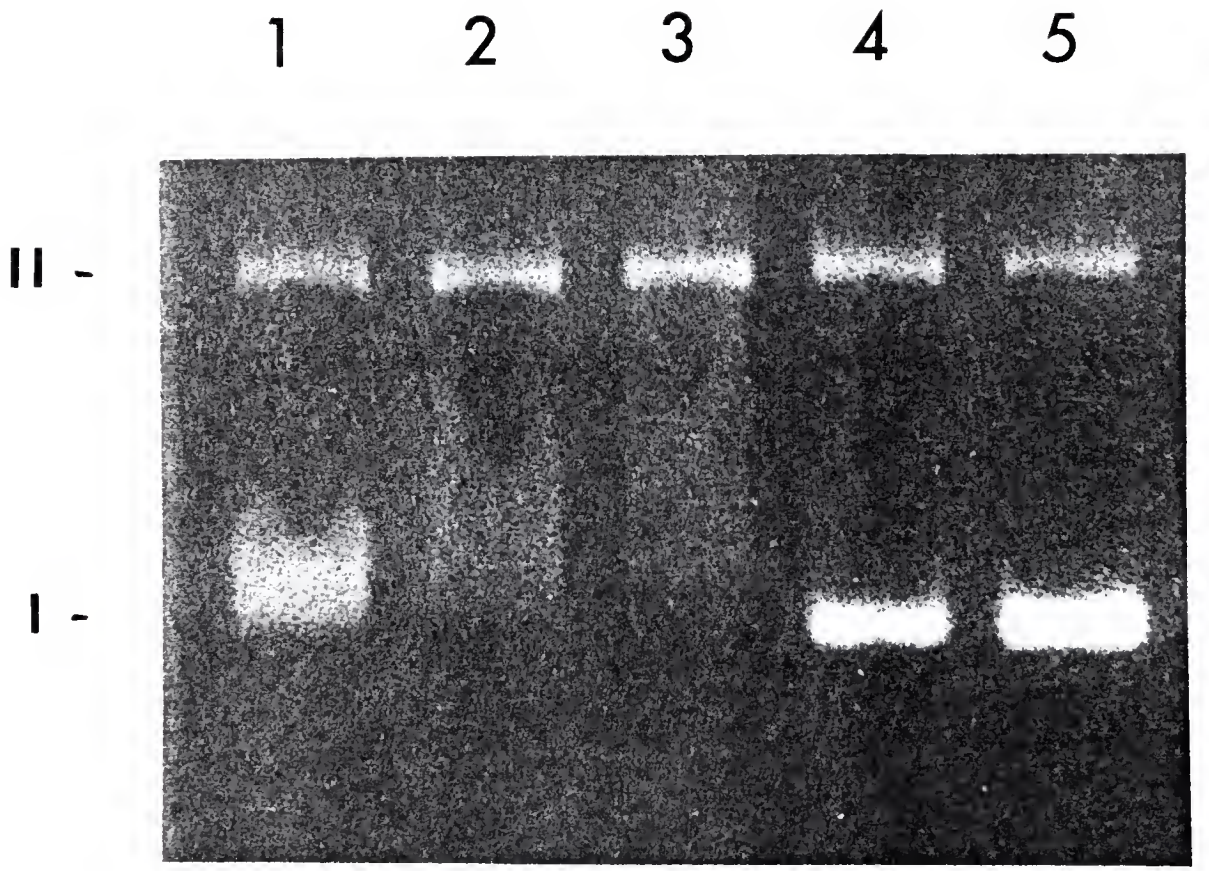

nmoles DNA Retained on Filter
1.0
0.5
5
10
15
20
Binding Protein (µl)

Figure 21

Agarose gel electrophoresis of DBP III - DNA mixtures. Reaction mixtures (0.05 ml) containing 2 nmol of øX174 DNA, 0.1 M potassium acetate and varying amounts of DBP III were incubated at 37°C for 10 min. Reaction mixtures were applied to a 1.4% agarose gel and electophoresed as described in Chapter II. Reaction mixtures contained 5 µl (lane 1), 10 µl (land 2), 15 µl (lane 3, 4) of DBP III (Fraction V). The reaction mixture in lane 4 was made 0.5% SDS before electrophoresis. Lane 5 contains a control reaction mixture without protein.


1
2
3
4
5
II -
I -

passed through nitrocellulose filters. The amount of DNA retained on the filters was the same whether the substrate was form I or form IV DNA (figure 22). It is possible that the form IV DNA was not totally relaxed and contained a small number of superhelical turns. Residual superhelicity could exist because the DNA binding reactions were performed under conditions different from those used to relax form I DNA with topoisomerase. Another method for examining the role of superhelicity in DNA binding is to relax form I DNA by intercalation with ethidium bromide. The intercalation of ethidium into DNA results in a reduction of primary helical turns in a covalently closed molecule (Bauer and Vinograd, 1968). At a critical concentration of ethidium, which is dependent upon temperature and ionic strength (Wang, 1969), the decrease in primary helical turns will cause form I DNA to have a superhelix density of zero. A further increase in the concentration of ethidium causes the form I DNA to become positively supercoiled. Form I DNA and increasing amounts of ethidium bromide were incubated with DBP III and filtered. As shown in figure 23 no change in the amount of DNA retained by DBP III was observed. As a control, the same experiment was performed using histone HI. HI protein has been shown to bind preferentially to covalently closed DNA providing the DNA is supercoiled (Vogel and Singer, 1975). The amount of DNA retained by HI was reduced ten fold at an ethidium bromide concentration of 0.3 μg/ml (figure 23). The experiments with form IV DNA and DNA relaxed by ethidium bromide suggest that

Figure 22

DBP III activity with form I or form IV DNA as substrate. DBP III reactions were as described in figure 20 except the DNA was 1 nmole of either form I DNA (o) or form IV DNA (▲). DNA binding activity was measured as described in Chapter II.

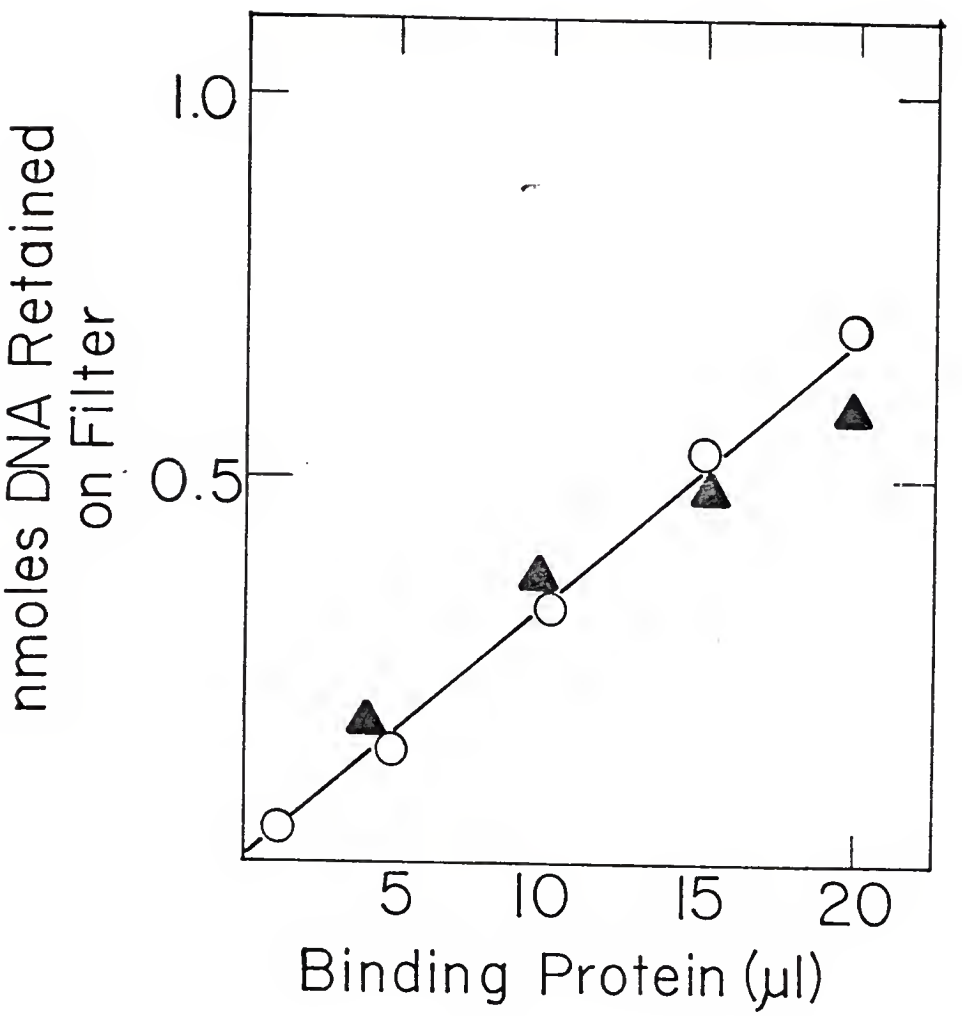
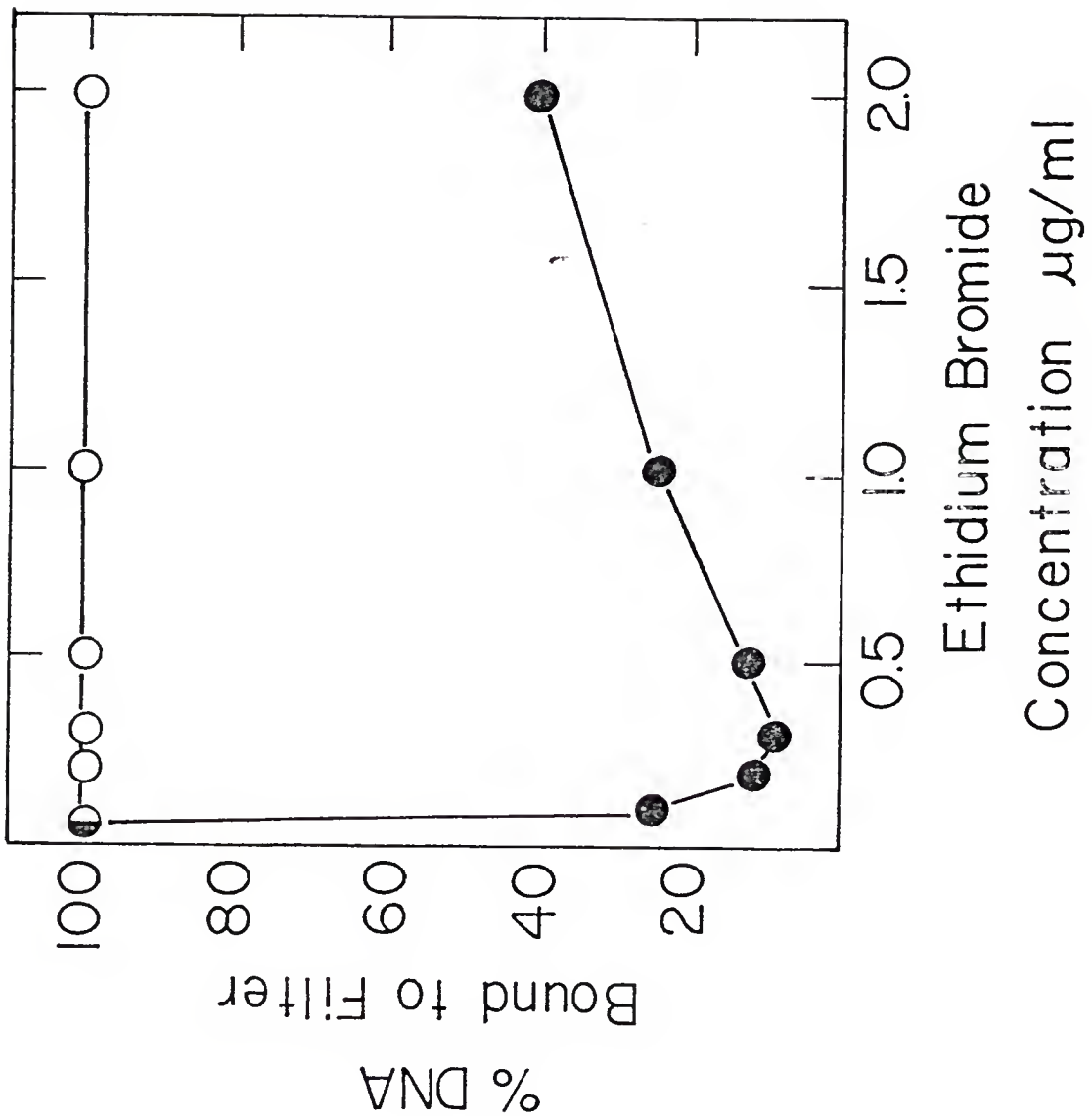

nmoles DNA Retained on Filter
1.0
0.5
5
10
15
20
Binding Protein (µl)

Figure 23

Effect of ethidium bromide intercalation on DBP III activity. Reaction mixtures (0.1 ml) containing 1 nmole of ØX174 [^{3}H] form I DNA, 0.1 M potassium acetate, 50 mM NaCl and the indicated concentration of ethidium bromide were preincubated at 37°C. Sufficient DBP III (o) or histone H1 (●) was added to bind about half of the input form I DNA in the absence of ethidium. After 10 min. at 37°C the reactions were diluted with 1 ml of 5 X SSC containing the same concentration of ethidium bromide as the individual reaction mix. Binding activity in the absence of ethidium was taken as 100%.


% DNA
Bound to Filter
100
80
60
40
20
0.5
1.0
1.5
2.0
Ethidium Bromide
Concentration µg/ml

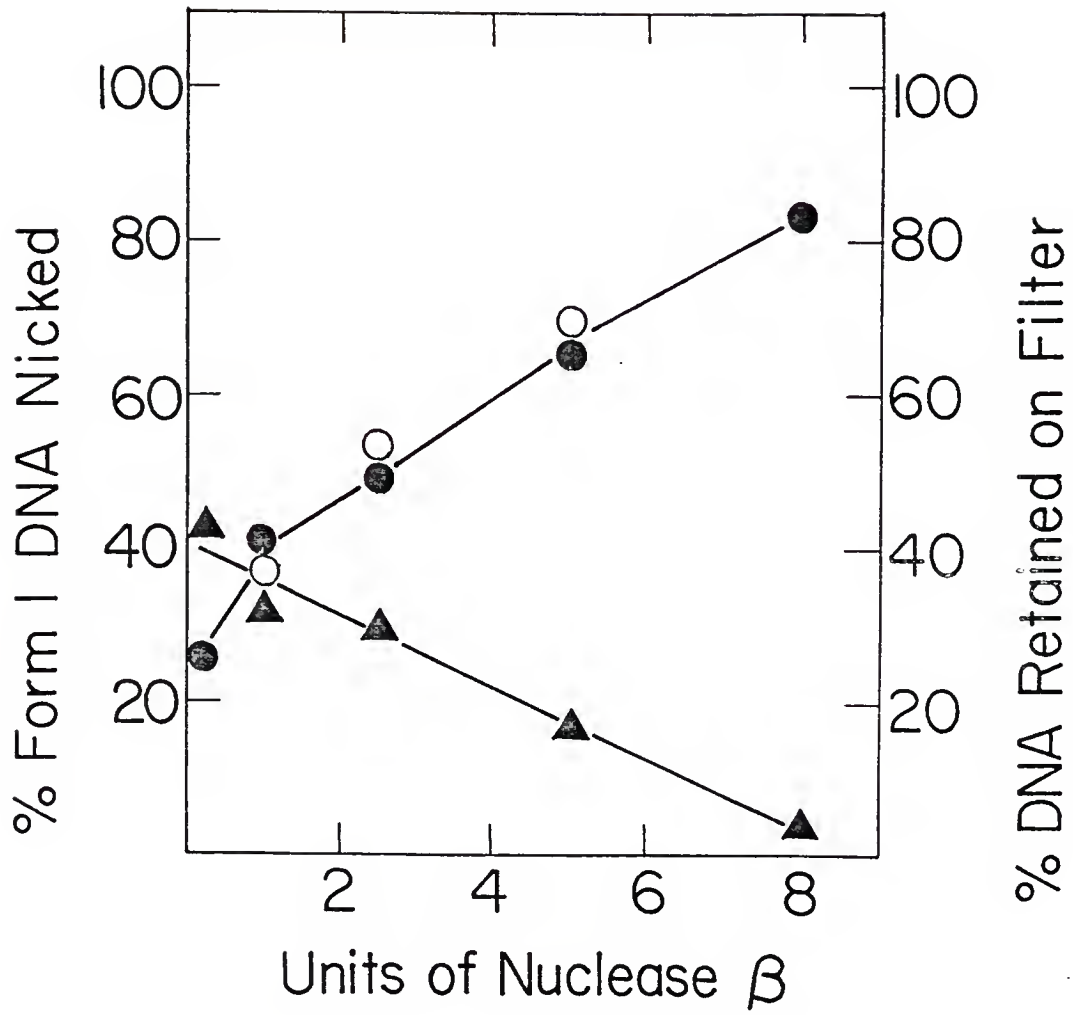
DBP III binds to covalenthy closed DNA regardless of the superhelix density.

DBP III forms a complex with covalently closed DNA that can be retained on nitrocellulose filters. No retention of DNA is observed if the molecule contains an interruption in one (form II) or both (form III) of the polynucleotide chains. What is the fate of the protein - DNA complex if the DNA is nicked after the complex is formed? DBP III was incubated with form I DNA and the samples were subsequently treated with increasing amounts of nuclease β. Protein - DNA complexes were measured by retention of DNA to a nitrocellulose filter. Nicking was monitored using the assay of Kuhnlein et al. (1976). Figure 24 shows that as form I DNA became nicked there was a comcommitant decrease in protein - DNA complexes retained by the filters. The introduction of a chain interruption appears to result in dissociation of the protein - DNA complex. Perhaps the protein can migrate on the duplex DNA and dissociate at an interruption in the helix. Alternatively, protein may induce an unknown secondary structure which cannot be maintained unless the DNA is covalently closed.

I wanted to examine the affinity of DBP III for single stranded polynucleotides and form I molecules other than øX174. Unlabeled polynucleotides were examined by performing binding competition experiments. A fixed amount of labeled form I DNA and varying amounts of unlabeled competitor polynucleotide were mixed and incubated with DBP III. Sufficient protein was added to bind 90% of the labeled form I

Figure 24

Effect of strand breakage on preformed DBP III - form I DNA complexes. Reaction mixtures (0.2 ml) containing 1.2 nmol of form I ØX174 [^{3}H] DNA, 0.1 M potassium acetate and 10 µl of DBP III (fraction V) were incubated at 37°C for 5 min. The samples were then treated with the indicated amounts of purified nuclease β for 10 min.at 37°C. Samples were split in half and assayed for either strand breaks (●) or DNA binding activity (▲) as described in Chapter II. The indicated amounts of nuclease β were also added to mixtures that did not contain any DBP III and the percentage of molecules with strand breaks was determined (o).


% Form I DNA Nicked
% DNA Retained on Filter
100
80
60
40
20
2
4
6
8
Units of Nuclease β

DNA in the absence of competitor DNA. Unlabeled øX174 form I DNA was used as a control. When the molar ratio of competitor to labeled DNA reached 1.0 there was a 50% decrease in labeled DNA retained on the filter (figure 25). Form II øX174 DNA was less effective then form I DNA in reducing retention of labeled DNA. At a molar ratio of 1.0 (competitor/labeled DNA) 80% of the label was still retained. Single stranded øX174 DNA at a molar ratio of 1.0 reduced the amount of label bound to 35%.

Since DBP III showed significant affinity for single stranded DNA I wanted to examine if there was any nucleotide specificity in DNA binding. [^{3}H] øX174 form I DNA was mixed with varying amounts of deoxy - homopolymers and incubated with DBP III as described in the previous experiment. Poly (dT) decreased the retention of labeled DNA to the same extent as øX174 single stranded DNA (figure 26). When poly [dA] was used as the competing polynucleotide there was no decrease in labeled DNA retained on the filter. Poly (dG) did compete with labeled form I DNA but to a lesser extent than the poly (dT). The differences observed between the homopolymers dG, dT, and dA in regard to competing for DBP III may reflect base specific binding or merely a preference for the most unordered polynucleotide tested, poly (dT) (see Chapter V for discussion). A DNA binding activity isolated from liver nuclei also demonstrates binding to single stranded polynucleotides (Comings and Wallack, 1978). The authors determined that purified nuclear matrix proteins bind

Figure 25

Binding competition with various forms of øX174 DNA. Reaction mixtures (0.1 ml) containing 0.1 M potassium acetate, 1 nmol of øX174 [^{3}H] form I DNA and the indicated amount of unlabeled competitor DNA were preincubated at 37°C. Sufficient Fraction V DBP III was added to bind 90% of the [^{3}H] form I DNA in the absence of competitor and this amount of binding was used as 100%. (Δ) form III DNA created by Pst I cleavage of form I øX174 DNA; (●) unlabeled øX174 form I DNA; (o) single stranded øX174 DNA.

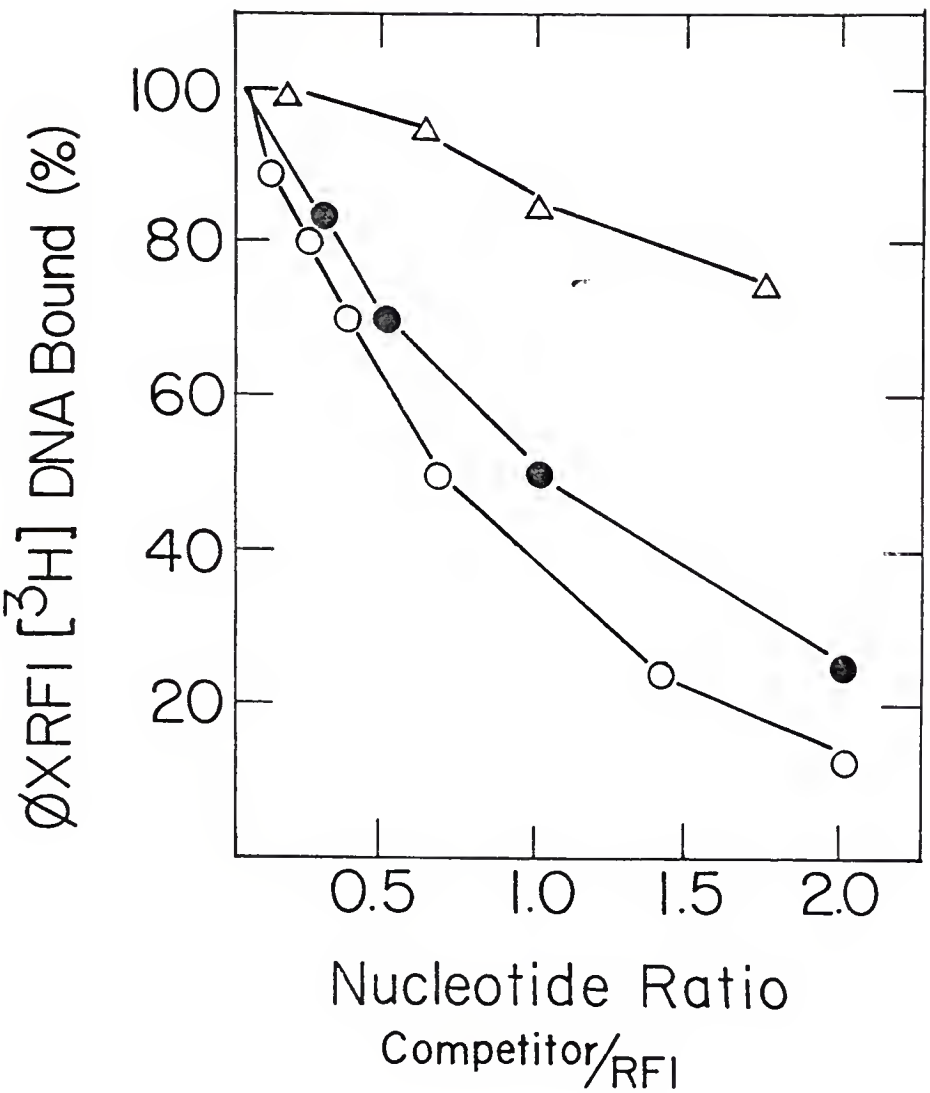
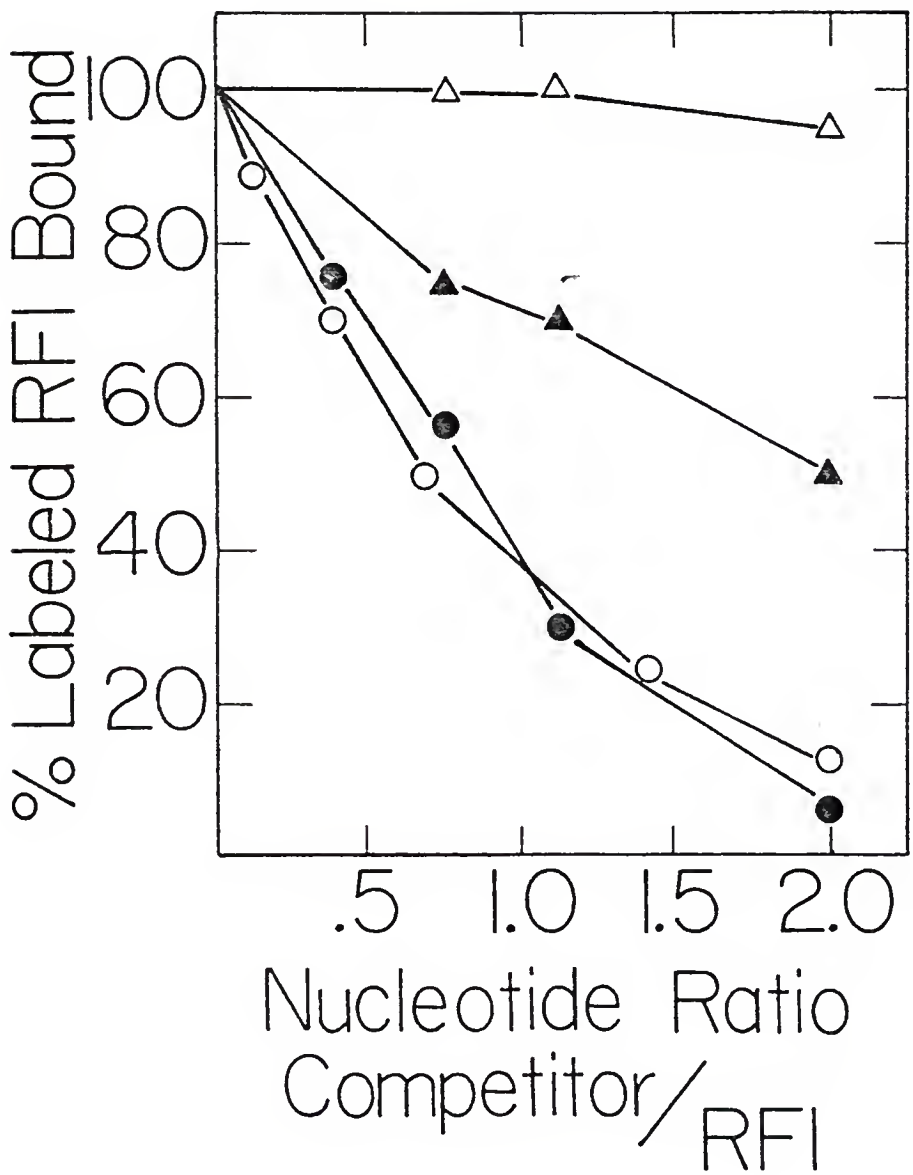

100
80
60
40
20
ØXRFI [3H] DNA Bound (%)
0.5
1.0
1.5
2.0
Nucleotide Ratio
Competitor/RFI

Figure 26

Binding competition with deoxyhomopolymers. Reaction mixtures containing øX174 [^{3}H] form I and various amounts of unlabeled competitor were incubated with Fraction V DBP III as described in figure 25. (Δ) poly [dA], (▲) poly [dG], (●) poly [dT] or (o) single stranded øX174 DNA were included prior to protein addition.

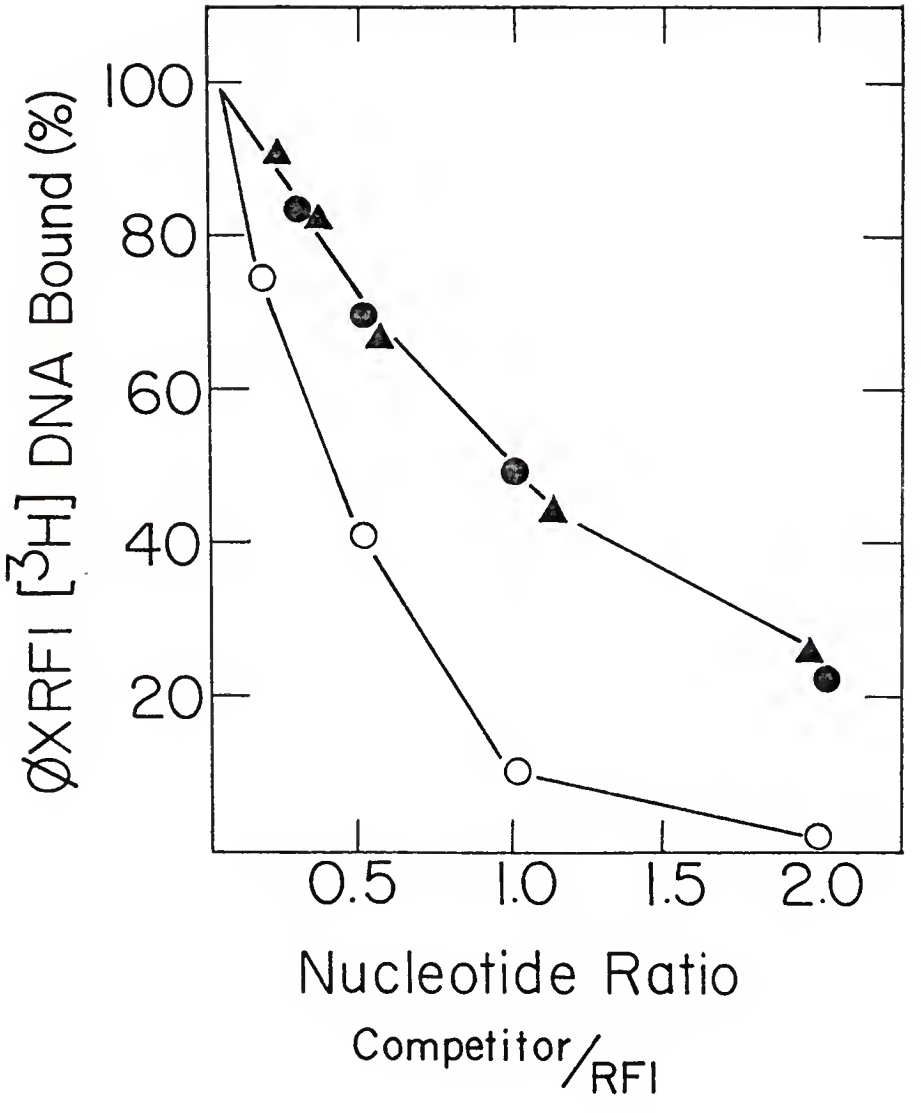

% Labeled RFI Bound
100
80
60
40
20
.5
1.0
1.5
2.0
Nucleotide Ratio
Competitor/RFI

preferentially to denatured DNA. Poly (dT) was found to be a better competitor than other homopolymers.

Proteins that bind specific DNA sequences have been isolated from both procaryotic and eucaryotic sources. A classical example is the binding of lac repressor to operator sequences of the E. coli lactose operon (Gilbert and Muller-Hill, 1970; Riggs et al., 1970). A protein fractionated from Drosophila embryos binds specifically to satellite DNA sequences of Drosophila melanogaster (Hsieh and Brutlag, 1979a). A single repeat unit of Drosophila satellite DNA (359 base pairs) has been cloned into the E. coli plasmid pBR322 (aDM 23-24). Both satellite DNA of Drosophila and the single satellite repeat cloned in pBR322 have been sequenced and 70% of the nucleotides are A or T (Hsieh and Brutlag, 1979b). The binding protein from Drosophila is similar to DBP III in that a threshold temperature is required for protein to bind DNA and protein - DNA complexes are not dissociated by high salt. Since the Drosophila binding protein and DBP III have some biochemical similarities I wanted to examine the relative affinity of DBP III for satellite DNA sequences from Drosophila. Varying amounts of plasmid DNA were mixed with labeled øX174 from I DNA and incubated with DBP III. Plasmid DNA containing the Drosophila satellite sequences (aDM 23-24) was a more effective competitor than either unlabeled øX174 DNA or form I pBR322 DNA (figure 27). Perhaps the Drosophila DNA contains a specific sequence that DBP III can recognize.

Figure 27

Binding competition with other form I DNA. Reaction mixtures, as described in figure 25, contained 1 nmole of øX174 [^{3}H] form I DNA and the indicated amounts of unlabeled competitor DNA. Sufficient DBP III was added to bind 90% of the labeled form I DNA in the absence of competitor DNA. (●) unlabeled øX174 form I DNA; (▲) form I pBR322 DNA; (o) form I aDM 23-24 DNA (a plasmid containing Drosophila satellite DNA). DNA binding activity was measured as described in Chapter II.


100
80
60
40
20
ØXRFI [3H] DNA Bound (%)
0.5
1.0
1.5
2.0
Nucleotide Ratio
Competitor/RFI

The affinity of DBP III for poly (dT) and the high A·T content of satellite DNA suggests the data reflects a nucleotide preference rather than a sequence specific binding.

The experiments presented above demonstrate that DBP III binds to covalently closed duplex DNA and single stranded DNA. The superhelix density of the covalently closed DNA molecule appears to have no effect on the amount of DNA bound by DBP III. The introduction of a chain scission results in dissociation of the protein - DNA complex. These results are discussed in the following chapter.

CHAPTER V
DISCUSSION

Two proteins from Ustilago maydis have been isolated and characterized. One protein is a nuclease, termed nuclease β, and this protein has been purified to about 70% homogeneity. Characterization of DNA hydrolysis by nuclease β and comparison with known nucleases from other sources suggest nuclease β may play a role in DNA replication or recombination. The other protein studied is a DNA binding protein, termed DBP III, and has the property of remaining bound to DNA in the presence of 2 M NaCl. DBPIII binds to covalently closed duplex DNA and single stranded DNA. This DNA binding activity from Ustilago shows some similarities to proteins extracted from the nuclear matrix of eucaryotic cells and a DNA binding activity from Drosophila embryos. The possible roles of DBP III in DNA metabolism are discussed below.

Characterization of Nuclease β

Nuclease β was purified 11,200 - fold from crude extracts and both physical and functional aspects of the enzyme were analyzed. Gel electrophoresis of purified nuclease β (fraction VI) suggested the preparation was 70% homogeneous and the polypeptide containing nuclease activity had a molecular weight of 68,000. Velocity sedimentation in glycerol gradients gave a sedimentation coefficient for nuclease β of

4.3 S. The molecular weight and shape of a protein determine its sedimentation coefficient (Martin and Ames, 1961). The average radius of a protein, termed the Stokes radius, can be determined by gel filtration (Siegl and Monty, 1966). Nuclease β had a Stokes radius of 36Å. A comparison of results from SDS-gel electrophoresis, velocity sedimentation and gel filtration indicates nuclease β exists in solution as a globular polypeptide with a molecular weight of 68,000.

Reaction Conditions

The nuclease was optimally active at pH 6.0 in 0.2 M sodium or potassium acetate buffer. Divalent cations (eg. Mg^{++} and Co^{++}) or chelating reagents such as EDTA had no effect upon nuclease β. The enzyme was strongly inhibited by nucleotides. ATP and dATP at 1 μM reduced nuclease activity 50%. SI nuclease of Aspergillus oryzae (Wiegand et al., 1975) and an endo-exonuclease from Neurospora crassa (Fraser et al., 1976) are examples of other eucaryotic nucleases that are inhibited by ATP.

Mode of DNA Hydrolysis by Nuclease β

Nuclease β is highly active on single stranded DNA. Denatured linear DNA is probably hydrolyzed by the enzyme in a 5' directed exonucleolytic manner. Three observations suggest nuclease β can cleave DNA exonucleolytically. First, a time course a denatured DNA hydrolysis was linear from the point of enzyme addition. Endonucleolytic cleavage would show an initial delay in production of acid soluble DNA because a cleavage event may produce an oligonucleotide that is still acid precipitable. Second, a partial digest of

DNA contained 30% of the product as mononucleotide. Random endonucleolytic digestion would not produce mononucleotides at an early stage of digestion. Third, ^{32}P-label was made acid soluble from [^{3}H] DNA labeled at its 5'-end with [^{32}P] PO_4 faster than the ^{3}H-label while the ^{32}P-label was not preferentially released from [^{3}H] DNA with a 3'-terminal ^{32}P. These three observations together suggest nuclease β hydrolyzes linear, denatured DNA in a 5' directed exonucleolytic manner. Single strand specific exonucleases have been isolated from both procaryotic and eucaryotic cells (for reviews see: Lehman, 1971; Kornberg, 1980).

Nuclease β appears to hydrolyze DNA in a distributive fashion. A [^{3}H] DNA digest was begun under conditions of DNA excess; all enzyme molecules should have been saturated with substrate molecules. Addition of unlabeled DNA caused an immediate decrease in the rate of [^{3}H] DNA becoming acid soluble. This experiment suggests nuclease β cleaves DNA in a random or distributive manner.

Nuclease β is also active on circular DNA. Both single and double stranded circular DNA can be cleaved by nuclease β. The cleavage of covalently closed, circular DNA occurs about 1,200 times slower than a strand scission event on denatured linear DNA. Thus nuclease β can cleave DNA in an endonucleolytic or exonucleolytic fashion. The recBC coded nuclease from E. coli is an example of an endo-exonuclease (Goldmark and Linn, 1972; Karu et al., 1973). Cells with mutations in recB or recC genes have an altered frequency of

recombination (Clark and Margulies, 1965; Willetts and Mount, 1969). Endo-exonucleases have also been isolated from Neurospora crassa (Fraser et al., 1976), Hemophilus influenzae (Friedman and Smith, 1972) and Vaccinia virus (Rosemond-Hornbeak and Moss, 1974).

When [^{3}H] DNA was made 100% acid soluble by nuclease β most (65%) of the product was recovered as mononucleotide. Enzymes that remove either 3' or 5' phosphates from mononucleotides were used to show that the mononucleotides generated by nuclease β contained a 3' phosphate. The mononulceotide products of digestion eluted from a Dowex column as the 3' isomers of deoxymononucleotides. The oligonucleotides produced by nuclease β were also terminated by 3' phosphates.

Nuclease β contains 5' nucleotidase activity and may hydrolyze RNA. The nucleotidase activity cosedimented and coelectrophoresed with the DNAse activity. Both DNAse and 5'-nucleotidase activities are optimally active at pH 6.0, unaffected by addition of EDTA and inhibited by ATP. A simple explanation is that a single active site on nuclease β can hydrolyze a 5' phosphate-nucleotide bond which exists as a phosphodiester or a phosphomonoester linkage. Another nuclease that contain both phosphomonoesterase and phosphodiesterase activity is nuclease P1 from Penicillium citrinum (Fugimoto et al., 1974). P1 cleaves nucleotides leaving a 5' phosphate and 3' hydroxyl. Exonuclease III of E. coli contains 3' phosphatase activity but only on polynucleotides (Richardson and Kornberg, 1964).

Nuclease β, like nuclease α of U. maydis (Holloman, 1973) is most active on single stranded DNA. The two enzymes differ with respect to optimal reaction conditions, mode of DNA hydrolysis, enzyme inhibitors and nucleotide products (Holloman, 1973; Holloman et al., 1981). Nuclease α is most active in Tris buffer at pH 8.0; nuclease β is inactive in Tris buffer. Nuclease α is totally inactive in the presence of EDTA whereas β is unaffected by EDTA. Nuclease β hydrolyzes linear DNA in a 5' directed exonucleolytic manner whereas nuclease α is a 5' and 3' directed nuclease that produces mainly short (2-4) oligonucleotide products. ATP severely inhibits nuclease β but nuclease α is unaffected by ATP.

Possible Roles for Nuclease β In vivo

Nucleases specific for single stranded DNA have been isolated from a variety of sources (Wiegand et al., 1975; Linn and Lehman, 1965; Gray et al., 1975; Kowalski and Laskowski, 1976). Nuclease β most closely resembles an enzyme isolated from Vaccinia virus (Rosemond-Hornbeak et al., 1974). The vaccinia nuclease hydrolyzes denatured DNA at pH 4.5. This viral nuclease is not affected by divalent cations or EDTA. The nuclease from vaccinia cleaves single stranded DNA endonucleolytically but produces mononucleotide products suggesting some exonucleolytic activity (Rosemond-Hornbeak and Moss, 1974). Both nuclease β and the vaccinia nuclease produce nucleotide products terminated by 3'-phosphoryl groups (K. I. Berns, personal communication).

Vaccinia DNA strands appear to be crosslinked at their ends (Berns and Silverman, 1970). The role of the vaccinia nuclease may be to nucleolytically separate the DNA strands allowing semiconservative replication of the viral DNA (Rosemond-Hornbeak and Moss, 1974). Parvoviruses (eg. adeno-associated virus or Minute virus of mice) are thought to require a nicking event near the DNA termini to allow replication of the viral genome to be completed (Tattersall and Ward, 1976; Straus et al., 1976; Hauswirth and Berns, 1977). The nicking of covalently linked DNA strands near their termini is part of a model proposed by Cavalier-Smith (1974) to explain the replication of eucaryotic DNA termini. A variety of single strand specific endonucleases have been isolated from mammalian cells (eg. see Wang et al., 1978) that possibly could cleave covalently linked termini. The similarities between nuclease β and the Vaccinia virus nuclease as well as the finding that some eucaryotic viruses have covalently linked DNA strands (see above) suggest nuclease β may be involved in chromosomal replication.

The hydrolytic properties of nuclease β suggest the enzyme may function in vivo by participating in some steps of recombination. The exchange of strands between duplex DNA molecules has been proposed to be a central feature of homologous recombination (Holliday, 1964). The recA gene product of E. coli has been found to catalyze strand exchange in vitro (Cunningham et al., 1980; Cassuto et al., 1980; West et al., 1981). Recent studies have suggested two

Prior to discussing the characterization of DBP III, I would like to review other DNA binding activities observed during the purification of DBP III.

Purification of DNA Binding Activities

Fractionation of cell extract by phosphocellulose or Sephacryl chromatography resulted in more than one peak of salt resistant DNA Binding activity. Activity not absorbing to phosphocellulose eluted from Sephacryl S-300 slightly ahead of bovie serum albumin (BSA). Subsequent fractionation of DNA binding activity not absorbing to phosphocellulose was unsuccessful. Since the DNA binding activity eluting from Sephacryl ahead of BSA showed no preference in binding form I over form II DNA the characterization of this binding activity was not pursued.

Activity absorbing to phosphocellulose eluted as two peaks of DNA binding activity when applied to Sephacryl S-300. A peak of DNA binding activity eluting just after a BSA marker coincided with a peak of nuclease activity. The nuclease was most active on single stranded DNA in the presence of a divalent cation (eg. Mg^{++} or Co^{++}). The nuclease activity eluting from Sephacryl is similar to the previously characterized nuclease α of U. maydis (Holloman et al., 1981). Both enzymes are optimally active on single stranded DNA at pH 8.0, inhibited by EDTA and can nick form I DNA. Purified nuclease α preparations can bind form I DNA in the presence of EDTA without nicking the DNA and these protein-DNA complexes are not dissociated by 1.5 M NaCl. The possibility

requirements for recA protein catalyzed strand exchange (Cunningham et al., 1980; Shibata et al., 1981). RecA protein will form joint molecules if one participating DNA molecule is partially single stranded and either DNA molecule contains a free end. The production of nicks or gaps in DNA has been shown to be recombinogenic in vivo (Benbow et al., 1974; Broker and Doermann, 1975; Howard-Flanders, 1975; Konrad, 1977). A nick or gap produced by nuclease β would contain a 3' phosphate termini. Enzymes thought to repair gaps (polymerases) or nicks (ligases) cannot act on 3' phosphate terminated DNA (Kornberg, 1980; Lehman, 1974). Therfore gaps or nicks created by nuclease β would be expected to remain longer, increasing the possibility that initiation of strand exchange would occur.

Nuclease β is a single strand specific endo-exonuclease that contains 5' nucleotidase activity. A comparison of the properties of nuclease β with previously isolated nucleases suggest nuclease β may be involved in some aspects of replication or recombination. Mutants of Ustilago maydis deficient in recombination or replication have been isolated (Holliday, 1975) and may shed light on the role of nuclease β in vivo.

Characterization of DBP III

A DNA binding activity, termed DBP III, was purified from crude extracts of U. maydis by assaying for protein dependent retention of DNA on nitrocellulose filters. DBP III - DNA complexes did not appear to dissociate in 2 M NaCl since DNA was still retained upon nitrocelluose filters.

that nuclease α preparations are contaminated by a DNA binding activity cannot be totally excluded. These results suggest nuclease α may be responsible for both the DNA binding and nuclease activities observed to coelute from Sephacryl behind BSA. Perhaps nuclease α-DNA complexes formed in the presence of EDTA could be used to examine the binding specificity of the nuclease. Although nuclease α is more active on DNA containing structural alterations such as those caused by base deamination (Holloman et al., 1981) no direct evidence exists that the nuclease binds or cleaves at these sites. Perhaps chemical crosslinking of nuclease α to structurally altered DNA would increase understanding of where nuclease α binds to the DNA molecule.

About 30% of the DNA binding activity absorbing to phosphocellulose excluded from Sehacryl and this activity is referred to as DBP III. A polypeptide of 47,000 d was the major protein observed when fractions containing DNA binding activity were analyzed by SDS-gel electrophoresis. Although the 47,000 d polypeptide could be the reduced subunit of a large protein the observed elution profile may be an artifact of ionic or hydrophobic aggregation. Another possibility is that binding activity is already complexed to DNA causing the protein to elute as a large molecule. However, polyethylene glycol phase partition and DEAE chromotography would be expected to remove most of the nucleic acids. Further studies are needed to define the polypeptide(s) responsible for DNA binding.

DNA Binding Properties of DBP III

DBP III binds optimally to DNA at pH 6.0. The addition of divalent metals or reducing agents does not affect the amount of DNA bound by protein. Binding occurs rapidly but requires a threshold temperature of approximately 25°C to observe protein binding to DNA. DNA binding proteins such as lac repressor (Riggs et al., 1970) or helix destabilizing proteins (Herrick and Alberts, 1976; Hosada and Moise, 1978) bind equally well at 4° or 37°C. Alternatively, a DNA binding activity isolated from Drosophila melanogaster demonstrates a temperature dependence for DNA binding (Hseih and Brutlag, 1979a). The DNA binding activity from Drosophila binds preferentially to DNA sequences found in complex satellite DNA of Drosophila. DBP III and the binding activity from Drosophila both form protein - DNA complexes only in low salt (less than 0.2 M NaCl) and once formed the complexes are stable in 2 M NaCl. Most DNA binding proteins (eg. lac repressor or helix destabilizing proteins) are removed from DNA by treatment with 2 M NaCl. Binding protein activity from both Ustilago and Drosophila is ablated by treating reactions with SDS (0.5%) or proteinase K before filtration.

The amount of form I retained on a filter was directly proportional to the amount of added protein. A two-fold increase in the amount of DBP III added caused a doubling in the amount of DNA retained on the filter. The approximate number of protein molecules bound to each DNA molecule can be calculated by making two assumptions. First the 47,000 d polypeptide is responsible for DNA binding. Secondly, the

47,000 d polypeptide represents 20% of the protein in DBP III preparations. If one makes the assumptions mentioned above then 1 μg (1.2×10^{13} molecules) of the 47,000 d polypeptide would bind 2 μg (3×10^{11} molecules) of øX174 form I DNA. This yields a value of 40 protein monomers per DNA molecule retained on the filter. The elution of DBP III from Sephacryl as a large molecule together with the approximation of protein monomers per DNA molecule suggests DBP III may be active as an aggregate of polypeptides.

DBP III binds form I DNA but not form II or form III DNA to nitrocellulose filters. Additionally, examination of DNA from reaction mixtures by agarose gel electrophoresis revealed that the mobility of form I DNA but not form II DNA was altered in the presence of DBP III. Therefore filter retention of DNA or gel electrophoresis of DBP III - DNA mixtures suggest DBP III binds preferentially to form I DNA.

Form I DNA differs from form II DNA in that form I DNA is covalently closed and negatively supercoiled. The role of superhelicity in DNA binding was examined by changing the superhelix density of covalently closed DNA used as substrate. Form I DNA was relaxed by treatment with Ustilago topoisomerase and then used as substrate for DBP III. The amount of DNA bound by DBP III was the same whether the substrate was form I or form IV DNA. A small number of superhelical turns may have been present in the form IV DNA because of the inability of the topoisomerase to totally remove the negative supercoils in form I DNA. Also, the

binding reaction was performed at 37°C while the relaxation reaction was done at 25° C. This temperature difference would be expected to add only about 1 superhelical turn per molecule of øX174 DNA (Wang, 1969). The equal binding of form I and form IV DNA by DBP III suggests that a decrease in superhelicity does not affect binding. Another method employed to examine the role of superhelicity in the binding of DBP III to DNA was to alter the superhelix density with the intercalating dye ethidium bromide. Intercalation of ethidium into covalently closed DNA removes primary helical turns in DNA (Bauer and Vinograd, 1968). The number of primary turns removed from a DNA molecule is dependent upon the concentration of ethidium, the temperature and ionic strength of the solution (Wang, 1969). When DBP III was incubated with form I DNA and increasing amounts of ethidium bromide there was no change in the amount of DNA retained on the filter. To demonstrate that ethidium had reduced the superhelix density of the DNA, binding reactions were repeated using histone H1 in place of DBP III. H1 binds preferentially to superhelical DNA (Vogel and Singer, 1975a) and removal of superhelical turns by ethidium reduces the amount of DNA bound (Vogel and Singer, 1975b) by H1. The binding of H1 to form I DNA was reduced 10-fold at an ethidium bromide concentration of 0.3 μg/ml. However a reduction in the superhelix density of a convalently closed molecule by addition of 0.3 μg/ml of ethidium bromide did not affect the amount of DNA bound by DBP III. These data suggest that superhelicity of

form I DNA may not be the reason DBP III shows a preference for form I DNA over form II or form III DNA.

Another possibility is that filter retention of form I DNA by DBP III occurs because the DNA molecule is covalently closed. The method used to determine the effect of a chain interruption on DBP III - DNA interactions was to nick DBP III - form I DNA complexes with a nuclease. Form I DNA was incubated with DBP III and then treated with nuclease β in order to introduce a limited number of nicks in the form I DNA. Reactions were assayed for strand breakage and DNA retention to filters. As the amount of form I DNA that was nicked by nuclease β increased there was a proportional decrease in the amount of DNA retained on the filters. Thus it appears that DBP III - DNA complexes dissociate if the DNA becomes nicked. Perhaps the binding of DBP III to DNA introduces an unknown secondary structure in covalently closed DNA that cannot be maintained if the DNA is nicked. The apparent wrapping of DNA around histones to form nucleosomes provides an example of protein induced secondary structure in DNA (Kornberg,1977). Alternatively DBP III may recognize a chain interruption directly and dissociate from the DNA. Dissociation of DBP III at a nick would require migration of DBP III along the helix until a chain interruption was encountered. Examples from the literature suggest protein movement on DNA requires ATP hydrolysis. The rep gene product of E. coli is a DNA dependent ATPase than can unwind duplex DNA (Scott and Kornberg, 1978; Kornberg et al., 1978; Duguet et al., 1978). A model proposed to explain the

catalyzed unwinding of the helix suggests rep protein uses ATP hydrolysis to induce a conformational change in the protein that forces the DNA to unwind (Das et al., 1980). DNA helicase I is an enzyme thought to migrate on DNA using ATP hydrolysis to both facilitate protein movement and unwind DNA (Abdel-Monem et al., 1976; Kuhn et al., 1978). Other enzymes thought to hydrolyze ATP for protein translocation along a DNA molecule include type I restriction enzymes (Yuan et al., 1980), recBC DNAse (Rosamond et al., 1979) and recA protein of E. coli (Cassuto et al., 1981). Although the mechanism by which ATP hydrolysis is couple to protein movement along DNA is not well understood most proteins thought to migrate on DNA hydrolyze ATP. A possible exception to ATP-driven protein movement on DNA is seen with sequence specific binding proteins. Lac repressor (Riggs et al., 1970) and type II restriction enzymes (P. Modrich, personal communication) show measurable association rates in excess of that expected if association with target sequences was limited by diffusion in solution. It has been suggested that lac repressor can migrate on the DNA (Riggs et al., 1970; Richter and Eigen, 1974). Diffusion of a protein on a DNA polymer would occur if electrostatic forces in protein - DNA binding are minimal (Schranner and Richter, 1978). A prediction of protein migration is that DNA fragment size would affect the association rate constant. Studies with the type II restriction enzyme Eco RI demonstrate a decrease in association

rate accompanies a decrease in fragment size of the DNA (P. Modrich, personal communication). Thus a possible model for the binding of DBP III to DNA would be that the protein diffuses on DNA and dissociates rapidly at a chain interruption.

Comparison of DNA Substrates by Binding Competition

The affinity of DBP III for form I DNA as compared to other DNA structures (eg. single stranded DNA or form III DNA) was examined by mixing labeled form I DNA with varying amounts of unlabeled polynucleotide. Unlabeled øX174 single stranded DNA was a more effective competitor for DBP III binding than unlabeled form I DNA. Radioactively labeled form II and form III DNA were not retained on filters by DBP III. When competition experiments between labeled form I and unlabeled form III were performed there was a measurable decrease in labeled DNA retained on the filter. Perhaps the affinity of DBP III for form III DNA is too low for protein - DNA complexes to be retained by filtration but sufficient to result in slight competition between DBP III and form I DNA. Alternatively if one assumes that DBP III migrates on DNA and dissociates at a nick then nicked DNA would not be retained on filters by DBP III but would compete for DBP III with form I DNA.

When single stranded øX174 was mixed with [^{3}H] øX174 form I DNA at a molar ratio of 1.0 and incubated with DBP III there was a 70% decrease in the amount of labeled DNA retained compared to a reaction containing only labeled form I DNA. I

examined the homopolymers dA, dT and dC to determine if DBP III bound perferentially to any specific base in a polynucleotide. Poly (dT) was as effective as øX174 single stranded DNA in competing against form I DNA while poly (dA) did not compete for DBP III to any appreciable extent. Poly dG was a less effective competitor than øX174 single stranded DNA. At a poly (dG) to form I [^{3}H] DNA ratio of 1.0 the amount of labeled DNA retained on the filter was reduced 30%. In the reaction conditions used above (0.1 M Na^+, 37°C) poly (dT) would be expected to exist as a random coil whereas poly (dG) and poly (dA) would contain a significant amount of secondary structure (Riley et al., 1966; Radding et al., 1962). Although DBP III may show nucleotide preference in binding to poly (dT) the relative inefficiency of poly (dA) and poly (dG) could result from poor binding of DBP III to polynucleotides containing extensive secondary structure.

Effective competition for binding of DBP III to form I DNA was observed using a plasmid containing satellite DNA from Drosophila. A single satellite DNA repeat of 359 base pairs was cloned into pBR322 (Hseih and Brutlag, 1979a) and sequenced (Hseih and Brutlag, 1979b). The cloned DNA sequence is 70% A-T and contains 5 runs of $(dA)_3$ $(dT)_3$. The form I plasmid containing Drosophila DNA may have been a more effective competitor than the parent plasmid (pBR322) because of the partial denaturation of A-T rich DNA at 37°C in 0.1 M potassium acetate. Another possibility is that the repeated deoxythymidylate residues present in the Drosophila sequence represent a preferred binding site for DBP III.

Possible Role of DBP III in Vivo

DBP III appears to bind covalently closed DNA and protein - DNA complexes are not dissociated by salt. DNA molecules containing a chain interruption are not effective substrates for DBP III. Endonucleolytic cleavage of preformed DBP III - form I DNA complexes appears to result in dissocation of protein - DNA complexes. Competition assays suggest a binding preference for single stranded øX174 DNA and the homopolymer poly (dT). Effective competition was also seen with a form I plasmid containing A-T rich sequences (aDM23-24). These binding characteristics observed for DBP III are similar to DNA binding proteins isolated from the nuclear matrix of mouse cells (Comings and Wallack, 1978). The nuclear matrix is a structure found in salt extracted nuclei that is associated with chromosomal DNA (Georgiev et al., 1978; Paulson and Laemmli, 1977; Berezney, 1979). Matrix proteins appear to hold chromosomal DNA in highly folded, negatively supercoiled loops (Ide et al., 1975; Benyajata and Worcel, 1976; Cook et al., 1976; Adolf et al., 1977). Recent experiments by Pardoll et al. (1980) suggest that active replication forks are physically associated with the nuclear matrix. Proteins isolated from the nuclear matrix bind DNA and are not dissociated by 2 M NaCl. Competition experiments suggest that matrix proteins have a binding preference for A-T rich DNA, single stranded DNA and the homopolymer poly (dT) (Comings and Wallack, 1978). The biochemical similarities between DBP III and matrix proteins

as well as the proposed involvement of the nuclear matrix in DNA replication (see above) suggest to me a possible role for DBP III *in vivo*. Pardoll *et al*. (1980) have suggested that DNA loops are passed through the nuclear matrix structure to be replicated. DNA entering the replication fork would have to be free of chain interruptions to avoid loss of an arm from the newly replicated chromosome. DBP III may be attached to the DNA entering the replication complex. The dissociation of DBP III upon encountering a nick in the DNA may cause the replication fork to stall or dissociate the DNA from the matrix complex. This would provide the cell with a mechanism for avoiding a potentially lethal event.

DBP III binds to covalently closed DNA and does not dissociate in 2 M NaCl. The amount of form I DNA bound by DBP III is not altered by changing the superhelix density of the DNA. DBP III - form I complexes dissociate rapidly if the DNA becomes nicked. A comparison of DBP III with other DNA binding proteins suggests DBP III may be associated with the nuclear matrix structure *in vivo*.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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